AN INTEGRATIVE, COMMUNITY-COLLABORATIVE APPROACH TO INVESTIGATING HEALTH, DIET, AND THE ORAL MICROBIOME IN ANCESTRAL AND DESCENDANT COAST TSIMSHIAN COMMUNITIES

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Anthropology
with a minor in American Indian and Indigenous Studies
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

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ABSTRACT

This project employs a novel, integrative approach to investigating the relationship between periods of sociocultural transition, diet, and the oral microbiome in the ancestral and descendant Coast Tsimshian communities of British Columbia, Canada. Over the past 6,000 years, the Coast Tsimshian have experienced two significant periods of social transition: increasing social complexity during the transition from the Middle Pacific period (3500-1500BP) to the Late Pacific period (1500-500BP) and 19th century European contact, with subsequent ongoing colonization and increasing industrialization. Archaeological evidence from burials and household structures at winter villages along the coastline of Prince Rupert Harbour indicate that as warfare and the accumulation of personal wealth increased, the social complexity of the ancestral community increased and individual status differentiation emerged. The communities were organized around large, stratified, lineage-based, multigenerational households, which likely controlled access to local food resources. This project integrates genomic, isotopic, and osteological data with community-held knowledge from the descendant Metlakatla First Nation to identify inter-individual variation in the diet and health of the ancestral Coast Tsimshian population related to status differentiation during this period of increasing social complexity. Using ancient bacterial DNA extracted from the dental calculus of the ancestral community, this project also characterizes the composition of the oral microbiome of this community, and examines variation in oral microbial diversity correlated with inter-individual variation in diet and oral health. Finally, analogous methods of data collection are used to examine diet, health, and the composition of the oral microbiome in the descendant community in comparison with the ancestral community. This paired Ancestor-descendant comparative research framework

facilitates an investigation of how the Coast Tsimshian oral microbiome has adapted to a marine-based subsistence lifestyle, and to what extent the ancestral microbiome has been retained by the descendant community, as they experience increasing industrialization within their traditional homeland.

ACKNOWLEDGEMENTS

I owe thanks to so many people who have helped and supported me throughout this process. My family and friends have been a tremendous source of strength, as has my advisor, Ripan Malhi. Thank you for always believing in me. My committee – Katherine Amato, Stanley Ambrose, and Jessica Brinkworth – provided critical guidance throughout the project, for which I am incredibly grateful. Thank you also to Jenny Davis for taking me under your wing – your advice and encouragement has been invaluable.

This project would not have been possible without the collaboration, participation, and intellectual contributions of the Metlakatla First Nation. Thank you so much for welcoming me on to your land, sharing your knowledge, and trusting me to work with the Ancestors. Special thanks to Barbara Petzelt and Joycelynn Mitchell for all of your hard work facilitating our visits to the community, as well as this project as a whole.

There are many others who provided intellectual and technical support for this research. Thank you to Jerome Cybulski for sharing your wealth of knowledge with me. Thanks are also due to the staff at the Canadian Museum of History who welcomed me in to the collections and provided support during my research visit. Thank you to Laura Weyrich and her team at the University of Adelaide, who provided training and support for key aspects of the microbial analyses in this project. Kacey Lohmann and Lora Wingate at the University of Michigan as well as Matthew Fort and Morgan Bailey at the University of Illinois for their assistance in analyzing the isotope samples for this project. I would also like to express my gratitude to Alvaro Hernandez, Chris Wright, and their team at the Roy J. Carver Biotechnology Center at the University of Illinois for providing phenomenal support for all of our genomic sequencing.

STATEMENT ON DATA ACCESS

The data collected from Ancestors and descendants of the Coast Tsimshian community was completed with permission from the Metlakatla First Nation and the Canadian Museum of History. Genomic sequence data, results of the osteological analyses, and metadata from descendant community members may be made available via data access agreement with Dr. Ripan Malhi at the University of Illinois at Urbana-Champaign.

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CHAPTER 1: INTRODUCTION

This project investigates how changes in human diet reflect periods of social transition, and how the oral microbiome mirrors these cultural changes. The research is part of decade-long collaboration between the Metlakatla First Nation and the Malhi Molecular Anthropology Lab within the Department of Anthropology at the University of Illinois at Urbana-Champaign. This project addresses the Metlakatla First Nation's interest in investigating dietary change as part of a broader community research initiative exploring the biological impact of European contact and ongoing colonization.

The Coast Tsimshian have occupied the coastline and islands surrounding what is now the port-town of Prince Rupert, British, Columbia, Canada (see Figure 1.1) for at least 6,000 years, based on genomic evidence (Cui et al. 2013; Lindo et al. 2017). During this time, the Coast Tsimshian experienced two significant periods of sociopolitical change which may have initiated dietary changes. In the Middle Pacific period (3500-1500BP), Coast Tsimshian villages increased in both size and social complexity, indicating a transition to a non-egalitarian society (Ames and Martindale 2014). Beginning in the 1800s, European settlement in this region, such as the Hudson Bay Company post of Fort Simpson, introduced new industries and a cash and wage economy. The commercialization of some traditional foods and disruption of traditional harvesting practices, paired with the introduction of processed market foods, has impacted the diet of Tsimshian descendants today (Anderson 2007).

The relationship between diet, biological health, and social transformation has been explored by anthropologists (Nguyen and Peschard 2003), but the emerging "anthropology of microbes" (Benezra et al. 2012) offers a new perspective. This project employs an integrative

approach which combines health data from osteological analyses and self-reported health histories from descendant community participants with stable isotope analyses of samples from both ancestral and descendant individuals and high-throughput metagenomic DNA sequence analysis of both ancient and modern human oral microbial DNA. Throughout the research process, data was presented to community representatives so community-held knowledge could be incorporated into interpretations. As such, this project provides an intellectually rigorous, holistic anthropological model for exploring the relationship between diet and the oral microbiome and demonstrates the benefits of integrating community-held knowledge into paleogenomic research. This approach has been used by archaeologists, including those working in Coast Tsimshian territory (Ames and Martindale 2014; Martindale and Marsden 2003; Martindale et al. 2017), but has not been well integrated into anthropological genetics research, where Indigenous knowledge is not given the same weight as genomic evidence (Bader and Malhi 2016; 2019). This is part of a history of contentious relationships between North American Indigenous communities and genomic researchers which has prevented both Indigenous communities from benefitting from genomic advances and researchers from exploring some areas of research interest (Malhi and Bader 2015). The community-driven, collaborative research framework modelled here begins to address the existing ascertainment bias in human genomic (Bader and Malhi 2016) and microbiome research (Ozga et al. 2016), where Indigenous communities from North America are underrepresented.

Investigating social change in the archaeological record

Much of the archaeological work investigating Coast Tsimshian culture draws on a processual framework focused on the circumstances under which social complexity and

inequality increased (Ames and Martindale 2014). Based on archaeological evidence from household structures, human burials, and grave goods across multiple village sites, the complex, highly stratified, lineage-based organization of Coast Tsimshian society emerged during the Middle Pacific period (3500-1500BP) (Archer 2001; Cybulski 2014; MacDonald and Cybulski 2001; Martindale 2003). By the start of the Late Pacific Period (1500-500BP), social structure in permanent winter villages had shifted abruptly from reflecting a society based on achieved status, likely through the collection of wealth, to a true non-egalitarian society (Kelly 2013) with individuals living within large, ranked, lineage-based, multigenerational households (Archer 2001). Several models have been used to explain this social change, and have primarily focused on how emerging elites could have leveraged resource specialization, control, and storage to drive their increasing status (Ames 1994; Ames 1981; Ames and Martindale 2014; Coupland 1988; Fladmark 1975).

Matson (1983; 1985) has suggested inequality arose as social entities like house-lineages emphasized their rights of ownership and control over specific food resource patches in response to population growth and increasing sedentism. Lineage ownership of resources such as berry patches and fishing grounds is noted in oral histories (Beynon 1980). This explanation is consistent with models of non-egalitarian foraging communities, in which families or social units control localized resources (Kelly 2013). These anthropological hypotheses, focused on control of local food resources, underscore the need for paleodietary reconstructions, like those proposed in this research project, to test these hypothesis. During this period of social transition in Coast Tsimshian history, one way developing social complexity may have manifested is in differential access to food resources or food preferences. This project is the first to utilize both apatite and collagen for isotopic reconstruction of paleodiet, as others working in the area have exclusively

discussed collagen data (Chisholm et al. 1983; Schwarcz et al. 2014). Reconstructing paleodiet using both apatite and collagen facilitates a more nuanced investigation of how increasing social complexity in Coast Tsimshian communities may have resulted in differential access to food resources, as it considers both whole-diet and dietary protein. Additionally, this project explores dietary variation between social groupings, instead of solely village-level comparisons, as previous researchers have done (Chisholm et al. 1983).

Building on this archaeological focus on social complexity, this research examines not the cause, but the impact of social transformation within the ancestral Coast Tsimshian community. Previous research has demonstrated dietary differences can mirror social inequality (Ambrose et al. 2003; Otero et al. 2015; Ubelaker et al. 1995); examining possible dietary differences and corresponding oral microbial communities provides an alternative perspective of the biological consequences of social inequality provided through macroscopic methods, such as those used in paleopathology (Armelagos and Cohen 1984; Crandall 2014). Socially-mediated access to food resources may have contributed to differential diet and food preferences between distinct social groupings, potentially influencing the taxonomic composition and diversity of the oral microbiome. Emerging anthropological literature is currently examining how the human microbiome may shape human evolution, as it influences the development and function of metabolic, immunologic, and neurologic processes, as well as disease susceptibility (Schnorr et al. 2016). Many "diseases of modernity" such as atherosclerosis and diabetes mellitus may be related to the dietary transition in human evolutionary history from a diverse hunter-gatherer diet to a modern high-carbohydrate diet in which most energy comes from grains, dairy, and refined fats and sugars (Lindeberg 2012). The human gut and oral microbiomes are being investigated as possible agents in susceptibility to these modern diseases (Thorburn et al. 2014).

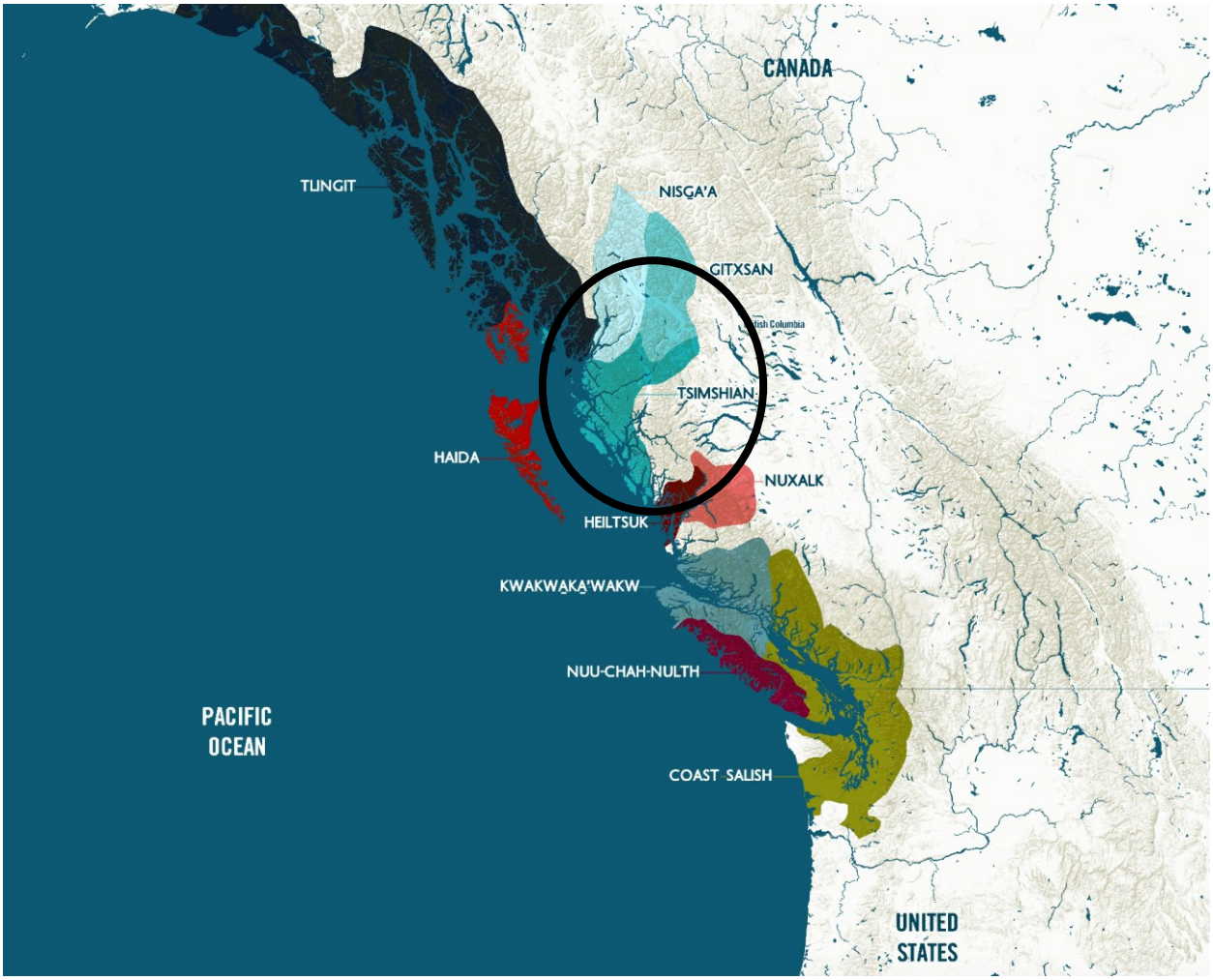

Figure 1.1. Map illustrating the approximate location of traditional Coast Tsimshian territory (circled in black and colored in dark blue), including the coast and islands between the Skeena and Nass Rivers in what is now British Columbia, Canada. Adapted from *amnh.org*.

Investigating social change in relation to European colonization and industrialization

Building on existing anthropological scholarship addressing the impacts of European colonization on Indigenous communities in the Americas (Klaus and Tam 2009; 2010; Larsen et al. 2001; Lindo et al. 2016), this project examines the relationship between diet, health, and the oral microbiome as one potential mechanism through which the cultural changes initiated by

European colonization have contributed to biological changes which result in negative health outcomes for descendant populations (Skelly et al. 2018). European colonization of the Americas undermined Indigenous food sovereignty and security, contributing to the health crises in which North American Indigenous communities experience higher rates of dietary-related health challenges like heart disease and diabetes mellitus (Turner and Turner 2008). Imbalances between the bacterial species of the oral microbiome have been implicated in these conditions (Scannapieco 2013; Slocum et al. 2016; Yeoh et al. 2013). Investigating changes in diet and the oral microbiome in association with increased industrialization allows for one possible assessment of how the ongoing cultural disruption experienced by First Nations communities may be embodied (Gravlee 2009), contributing to health disparities between these communities and their non-Indigenous counterparts.

Other researchers investigating the relationship between human diet and subsistence lifestyle and microbiome composition have utilized several approaches: analyzing the microbiome of living communities with non-industrialized lifestyles as a model for past human populations (Clemente et al. 2015; Obregon-Tito et al. 2015); assessing the microbiome of living Indigenous communities in North America (Ozga et al. 2016; Sankaranarayanan et al. 2015); or comparing the microbiome of ancestral and living populations without examining the genetic relationship between the two populations (Adler et al. 2013; Tito et al. 2012). In contrast, this project compares analogous data collected from both Coast Tsimshian Ancestors and a descendant community, a methodology which is facilitated by the collaborative research relationship with the Metlakatla First Nation. This research strategy reflects an entanglement framework (Martindale 2009) which examines the oral microbiomes of the ancestral and descendant Coast Tsimshian communities within the context of a broader political history,

encompassing both pre-European contact changes in social complexity and the sociopolitical changes initiated with European contact (Ames and Martindale 2014). Rather than treating the pre- and post-European contact populations as distinct entities, an entanglement perspective acknowledges previous research which has demonstrated the genomic and cultural continuity between these two communities over the past 6,000 years, and continuity with other Indigenous communities in the Pacific Northwest Coast region for at least 10,000 years (Cui et al. 2013; Lindo et al. 2017). Investigating the relationship between diet and the oral microbiome in an ancestral and descendant community allows for an analysis with greater time-depth and rigor than using lifestyle proxies or populations with unknown genetic relationships. This contextualizes the discussion of microbial taxonomic changes within the recent evolutionary history and adaptations of the Coast Tsimshian communities.

Organization

In the first data chapter (Chapter 2), a combination of osteological, genomic, and isotopic methods are used to examine inter-individual variation in diet and health. This variation is used to test whether individual-level indications of social complexity, such as individual status differentiation and skeletal and dental health disparities, are reflected in the diet of the Coast Tsimshian community during the Middle Pacific to Late Pacific period. These analyses investigate how individuals within the ancestral Coast Tsimshian community were impacted by the transition to a complex, highly stratified, non-egalitarian social organization.

Chapter 3 builds on these results to examine how inter-individual variation in diet and health is reflected in the ancestral Coast Tsimshian oral microbiome. Dental calculus samples from each of the 45 Ancestors analyzed in the previous chapter were sequenced to reconstruct

the ancestral oral microbiome. This chapter discusses how the fisher-hunter-gatherer subsistence strategy utilized by the ancestral Coast Tsimshian community has shaped the composition of the oral microbiome.

In the final data chapter (Chapter 4), analogous data on health, diet, and the oral microbiome from 17 members of the descendant Metlakatla First Nation are presented. The variation in diet, health, and the oral microbiome within this community is explored. In addition, the oral microbiome of the descendant community is compared to the ancestral Coast Tsimshian microbiome to elucidate how changes in diet and health related to ongoing colonization and increasing industrialization have impacted oral microbial taxonomic composition.

CHAPTER 2: RECONSTRUCTING COAST TSIMSHIAN DIET AND HEALTH IN THE CONTEXT OF INCREASING SOCIAL COMPLEXITY DURING THE MIDDLE-LATE PACIFIC PERIOD, 3500-500BP

Abstract

The ancestral Coast Tsimshian community from the area surrounding what is now Prince Rupert Harbour, British Columbia, Canada is well known for both its complex, highly stratified social organization and what archaeologists have deemed an "extreme" specialization in the consumption of salmon. This paper evaluates variation in diet and health as it relates to increasing social complexity during the transition from the Middle Pacific (3500-1500 BP) to the Late Pacific (1500-500 BP) periods. The use of stable isotope analysis introduces new data representing individual-level diet in this population. Carbon and nitrogen isotopes extracted from tooth root dentine provide estimates of the proportion of marine resources contributing to both whole diet and dietary protein. Additionally, osteological data provide insight into the impact of the high marine protein diet on oral health. This analysis will provide essential insight into the relationship between fisher-hunter-gatherer diet and health during a period of increasing social complexity on the Pacific Northwest Coast.

Introduction

The Coast Tsimshian have occupied the coast and offshore islands of the northern Pacific Northwest Coast for at least 6,000 years, according to genomic evidence (Lindo et al. 2017). Their traditional territory, which lies between the Nass and Skeena Rivers surrounding the modern port-town of Prince Rupert in what is now British Columbia, Canada, is an ecologically

rich environment combining coastal and off-shore marine resources with those from adjoining terrestrial and riverine environments. Coast Tsimshian society is distinct for having developed a highly stratified, non-egalitarian society without a coinciding shift to agricultural subsistence. The ability to move between these environments and exploit different seasonal resources may have facilitated increasing social complexity while maintaining a fisher-hunter-gatherer subsistence strategy.

Prior to European colonization, the ancestral Coast Tsimshian community comprised multiple villages where the Coast Tsimshian gathered during the winter (Figure 2.1), while subsisting off primarily marine resources (MacDonald and Cybulski 2001). During the Middle Pacific period (3500-1500 BP) these villages increased in social complexity (Martindale 2003). Archaeological evidence from burials indicate population size and social complexity increased during the latter half of the Middle Pacific period (2500-1500 BP) (see Archer 2001 for discussion). Variation in the inclusion of grave goods and evidence for expanded regional trade networks suggest the beginning of individual status differentiation (MacDonald and Cybulski 2001). A survey of skeletal trauma suggests warfare was widespread in the Prince Rupert Harbour region from 3000-1000BP (Cybulski 2014), and may have been a way to acquire the wealth driving this social transition (Ames 2001; Archer 2001). Analysis of household structures suggests the social structure in the permanent winter villages along the coastline shifted abruptly in 1500BP from a society based on achieved status, likely through the collection of wealth, to a true non-egalitarian society (Kelly 2013) with individuals living within the large, ranked, lineage based, multigenerational households (Archer 2001).

One way developing social complexity during the Middle Pacific period may have manifested is in socially mediated differential access to food resources or food preferences

between village communities, House-lineages, and/or individuals. A defining characteristic of non-egalitarian foraging communities is the control of localized resources by family or social units (Kelly 2013). Consistent with this expectation, lineage ownership of resources like berry patches and fishing grounds is documented in transcribed Coast Tsimshian oral histories (Beynon 1980) and ethnographic reports (Kan 1986). In their faunal analysis of the village site McNichol Creek, Coupland et al. (1993) note the absence of sea mammal and eulachon (*Thaleichthys pacificus*) remains, and suggest the House-lineages based in McNichol Creek were not as wealthy as those of other village sites, like Boardwalk (Ames 1998), and thus did not have their own eulachon fishing territory or hunting sea canoes.

While it is well documented that access to food resources was socially-mediated (Sobel et al. 2006), little biological evidence has been used to test the possibility that differential access to food resources and food preferences resulted in unique dietary profiles between individuals. The connection between social stratification and restricted access to dietary resources expected in non-egalitarian society (Kelly 2013) suggests there should be differences in dietary composition or breadth between individuals of different status, or individuals living in villages with hypothesized status differences. Additionally, the abrupt shift in household structure at the beginning of the Late Pacific period suggests there might be increased dietary variability between individuals from the Middle Pacific and Late Pacific periods, corresponding with the increased social stratification documented in the archaeological record.

In this paper, the relationship between diet and social complexity is explored by reconstructing the diet of Coast Tsimshian Ancestors via stable isotope analysis. Isotopic analysis of human tissues, such as tooth root collagen and apatite, facilitates the reconstruction of individual dietary profiles (Ambrose 1990; Ambrose and Norr 1993; Krueger and Sullivan 1984;

Lee-Thorp et al. 1989). This comparisons to be drawn between individuals from different inferred social status groups and villages sites to examine how social complexity drives dietary variation in the ancestral Coast Tsimshian community.

Relatedly, the impact of social complexity on diet has been implicated in health inequalities observed in the archaeological record (Ambrose et al. 2003, for example). Increasing social stratification, accompanied by social mediated access to food sources of varying nutritional quality, have been correlated with skeletal indicators of health inequality such as increased prevalence of anemia-associated skeletal lesions and dental pathologies (Cohen 1998). However, much of this work has been done in populations with evidence for agricultural intensification (Armelagos and Cohen 1984). Whether this association between social stratification, dietary variation, and health inequality remains in the context of a fisher-hunter-gatherer population has not been thoroughly investigated. Therefore, in addition to analyzing how increasing social complexity drives diet within the ancestral Coast Tsimshian community, skeletal and dental indicators of health will also be examined to investigate how social complexity impacts the health experiences of these individuals.

Results

A total of 45 Ancestors were sampled from two previously excavated village sites in the Prince Rupert Harbour region, Gb-To-31 “Boardwalk” (n=25) and Gb-To-33 “Lachane” (n=20) (Figure 2). The sampled Ancestors (Table 2.1 and Figure 2.1) include individuals from throughout the Early to Late Pacific periods (5500-500BP), estimated via a combination of radiocarbon dates and field notes on burial context (Cybulski, pers. comm.). Only individuals with third molars in occlusion were included, to ensure dental maturity of the sample group. The

sex of each individual was previously estimated by Cybulski (2014) based on skeletal morphology. This estimate was confirmed via assessment of crania morphology (Buikstra and Ubelaker 1994). The sampled Ancestors include 22 females and probable females, and 23 males and probable males. Additionally, some individuals (n=13, 28.9%) had evidence of labret wear (Cybulski 2015). Usually made of stone or wood, labrets are spool-shaped lip plugs worn throughout the Northwest Coast. Ethnographically, labrets are described as being worn by only women to denote free-status (as opposed to being enslaved) (Ames 1994). However, the archaeological record indicates prior to the period of European contact labrets were worn by select individuals of both sexes (Cybulski 1974; 1994). Thus labret wear was used as a proxy for higher status in this sample of Ancestors.

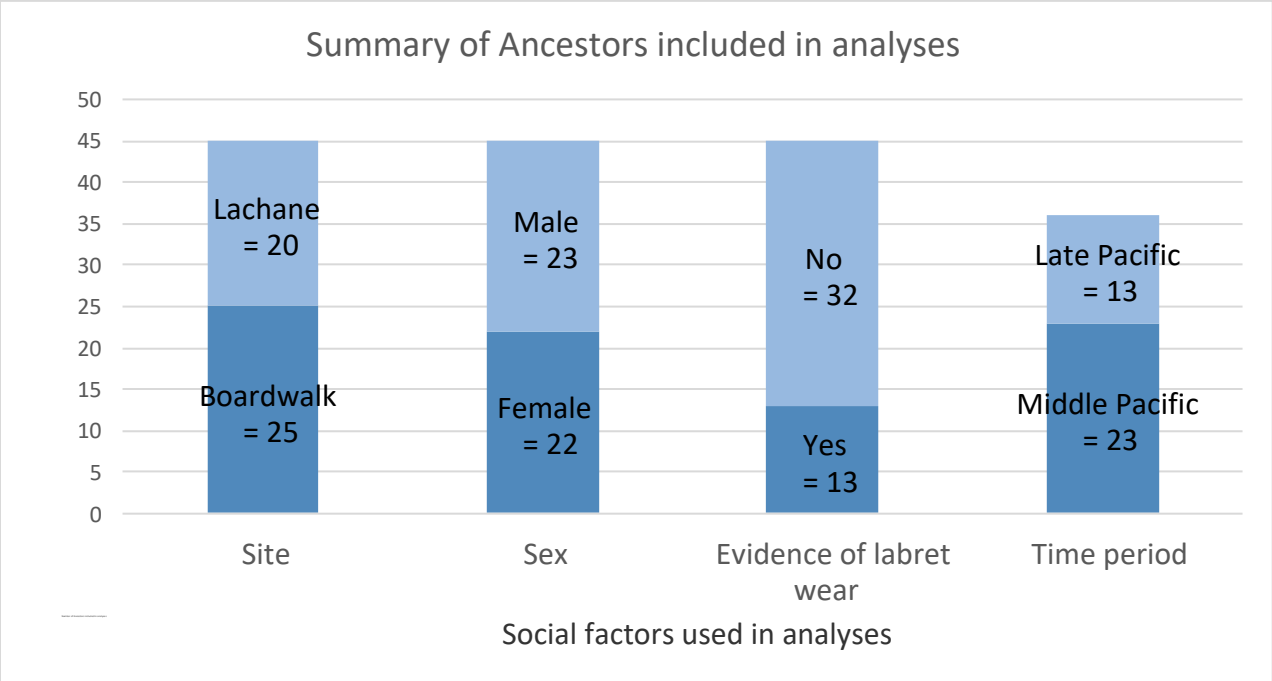

Figure 2.1. Summary of social groupings used to analyze osteological and isotopic data. All Ancestors included in the study were attributed to one of two "site", "sex", and "labret wear" groupings. Nine individuals were not included in analyses using "time period" because they could not confidently be assigned to either the Middle Pacific or Late Pacific periods using a combination of radiocarbon dating and analysis of excavation records.

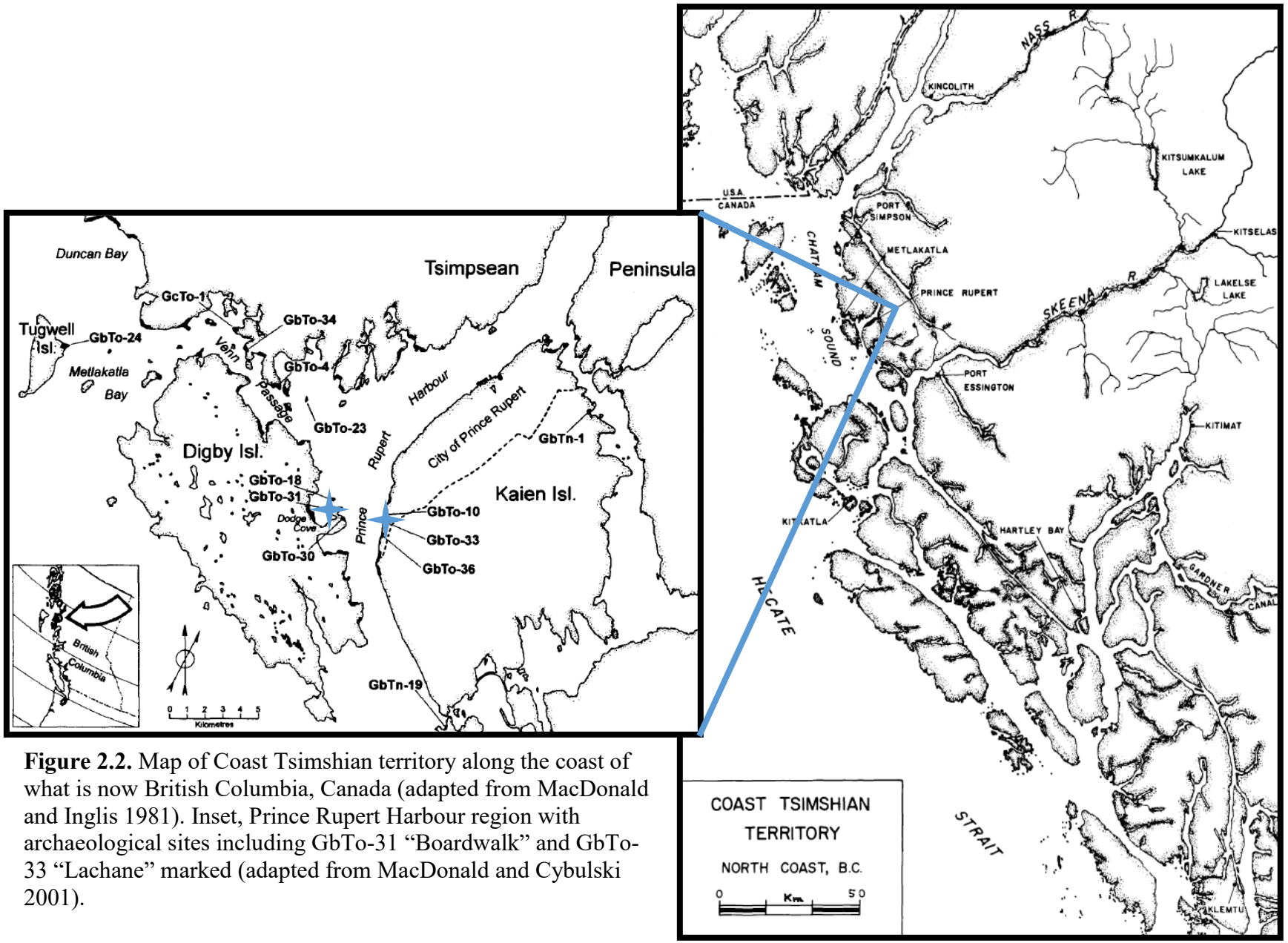

Figure 2.2. Map of Coast Tsimshian territory along the coast of what is now British Columbia, Canada (adapted from MacDonald and Inglis 1981). Inset, Prince Rupert Harbour region with archaeological sites including GbTo-31 "Boardwalk" and GbTo-33 "Lachane" marked (adapted from MacDonald and Cybulski 2001).

Table 2.1. Ancestors included in analyses				
Individual identifier	**Site**	**Estimated sex**	**Evidence of labret wear**	**Time period (Pacific)**
XVII-B-312	Boardwalk	Male	X	Middle
XVII-B-319	Boardwalk	Male	X	Middle
XVII-B-321	Boardwalk	Female		Middle
XVII-B-335	Boardwalk	Female		Late
XVII-B-339	Boardwalk	Female		Middle
XVII-B-343	Boardwalk	Female		Middle
XVII-B-347	Boardwalk	Male (possible)	X	Middle
XVII-B-360	Boardwalk	Female		Middle
XVII-B-364	Boardwalk	Male (possible)	X	Middle
XVII-B-378	Boardwalk	Female		Middle
XVII-B-380	Boardwalk	Male (possible)	X	Middle-Late
XVII-B-382	Boardwalk	Male (possible)	X	Middle
XVII-B-385	Boardwalk	Male		Unknown
XVII-B-387	Boardwalk	Female		Late
XVII-B-388	Boardwalk	Female		Early
XVII-B-406	Boardwalk	Female		Middle
XVII-B-409	Boardwalk	Male (possible)		Middle
XVII-B-410	Boardwalk	Male		Middle
XVII-B-411	Boardwalk	Female		Middle
XVII-B-412	Boardwalk	Female	X	Late
XVII-B-446	Boardwalk	Female (possible)		Late
XVII-B-447	Boardwalk	Male (possible)		Late
XVII-B-450	Boardwalk	Female		Middle
XVII-B-453	Lachane	Male		Middle
XVII-B-455	Lachane	Male		Middle
XVII-B-458	Lachane	Male (possible)		Middle-Late
XVII-B-459	Lachane	Female	X	Late
XVII-B-461	Lachane	Male (possible)		Middle-Late
XVII-B-463	Lachane	Male (possible)		Middle
XVII-B-473	Lachane	Female		Unknown
XVII-B-474	Lachane	Male		Middle-Late
XVII-B-475	Lachane	Male (possible)	X	Unknown
XVII-B-476	Lachane	Female		Middle
XVII-B-489	Lachane	Female (possible)		Middle
XVII-B-495	Lachane	Male (possible)	X	Late
XVII-B-496	Lachane	Male		Middle
XVII-B-501	Lachane	Female		Late
XVII-B-502	Lachane	Male (possible)	X	Middle-Late
XVII-B-504	Lachane	Female		Late
XVII-B-520	Boardwalk	Female (possible)		Late
XVII-B-525	Boardwalk	Male (possible)	X	Late
XVII-B-886	Lachane	Male (possible)		Late
XVII-B-889	Lachane	Female		Late
XVII-B-892	Lachane	Male (possible)	X	Middle
XVII-B-893	Lachane	Female (possible)		Middle

Osteological analyses

Each Ancestor was macroscopically examined for evidence of oral pathologies including carious lesions, alveolar abscesses, antemortem tooth loss, and periodontitis. Because of their relationship to anemia (Walker et al. 2009), presence/absence of cribra orbitalia and porotic hyperostosis was also recorded.

Of the 45 Ancestors analyzed, only 3 individuals (6.7%) had any observable carious lesions. However, 32 individuals (71.1%) had at least one active abscessed dental socket at their time of death. An ANOVA indicates there is no significant difference in the mean percent of abscessed sockets between the two sites ($p=0.875$), sexes ($p=0.890$), or between the Middle and Late Pacific periods ($p=0.901$). A Welch ANOVA indicates the difference in mean percent of observable sockets abscessed is approaching statistical significance ($p=0.074$) between individuals with and without evidence of labret wear. Individuals with evidence of labret wear had an average of 13.46% observable sockets abscessed. Individuals without labret wear had a mean of only 8.22% of observable sockets abscessed.

Half of the Ancestors analyzed (n=24, 53.3%) had one or more teeth lost antemortem (AMTL). An ANOVA indicates there is no significant difference in percent of AMTL by site ($p=0.320$), sex ($p=0.559$), labret wear ($p=0.708$) or time period ($p=0.524$).

Thirty Ancestors (66.7%) had intact alveolar bone at the second molar to be evaluated for periodontitis. Of these individuals, 93.3% (n=28) exhibited alveolar changes consistent with periodontitis. The severity of periodontitis was scored according to Buikstra and Ubelaker (1994). The majority of observable Ancestors (n=19, 63.3%) exhibited low severity periodontitis. A likelihood-ratio chi-square test indicates the severity of periodontitis exhibited

by individuals did not vary significantly by site (p=0.110), sex (p=0.922), labret wear (p=0.368) or time period (p=0.788).

Twenty-eight Ancestors had at least one orbital roof present to evaluate for cribra orbitalia. Of these individuals, 60.7% (n=17) presented skeletal lesions consistent with cribra orbitalia (healed or active). A likelihood-ratio chi-square test indicates the proportion of individuals with cribra orbitalia did not vary significantly by site (p=0.290), sex (p=0.934), labret wear (p=0.318) or time period (p=0.481).

Thirty-five Ancestors had sufficient preservation of the external cranial vault to evaluate the presence of porotic hyperostosis. Of these individuals, 74.3% (n=26) had skeletal lesions consistent with porotic hyperostosis. A likelihood-ratio chi-square test indicates the difference in prevalence of porotic hyperostosis between sites is approaching significance (p=0.059). At Boardwalk, 85.7% (n=18) of observable Ancestors had skeletal lesions consistent with porotic hyperostosis, whereas only 57.1% (n=8) of individuals at Lachane exhibited parietal lesions. A significant difference in prevalence was not observed between the sexes (p=0.627), labret wear (p=0.619), or time periods (p=0.448).

Isotopic analyses

Both collagen and apatite were successfully purified from the whole tooth root extracted from each Ancestor in the study. A summary of the results is presented in Table 2.2. Apatite percent yield ranged from 44.4 to 68.7% across the sample. Collagen percent yield varied from 7.5 to 22.9%. The expected atomic ratio of carbon to nitrogen (C:N) in collagen is 3.21 (Ambrose 1993). The C:N ratio of collagen extracted from these individuals ranges from 3.1 to 3.2, indicating a low likelihood of environmental isotope contamination (Ambrose 1993).

Table 2.2. Isotope values obtained from tooth roots, with quality measures										
Individual identifier	Dentine collagen						Dentine apatite			$\Delta^{13}C$ ap. - coll.
	Wt % yield	Wt % C	Wt % N	C:N	$\delta^{15}N$	$\delta^{13}C$	Wt % yield	Wt % C	$\delta^{13}C$	
XVII-B-312	20.1	37.0	13.5	3.2	19.2	-13.3	54.1	0.9	-10.9	2.5
XVII-B-319	16.6	41.4	15.1	3.2	19.3	-12.8	59.6	1.0	-10.0	2.7
XVII-B-321	17.6	40.3	14.9	3.2	19.7	-13.3	53.3	0.9	-11.0	2.3
XVII-B-335	16.5	45.7	16.8	3.2	18.5	-12.4	63.6	0.6	-10.3	2.1
XVII-B-339	15.5	37.0	13.5	3.2	20.1	-13.3	60.0	1.0	-10.5	2.8
XVII-B-343	11.4	43.6	15.8	3.2	18.1	-12.5	62.5	1.0	-9.8	2.7
XVII-B-347	21.5	38.3	14.1	3.2	19.8	-13.1	49.5	1.0	-10.0	3.1
XVII-B-360	13.5	45.2	16.5	3.2	19.6	-12.0	61.6	0.9	-8.8	3.2
XVII-B-364	16.7	38.3	14.1	3.2	19.7	-12.6	59.8	1.6	-9.5	3.1
XVII-B-378	15.9	37.8	14.0	3.2	19.7	-12.9	58.6	1.1	-10.0	2.9
XVII-B-380	16.8	42.0	15.5	3.2	19.2	-13.0	50.7	1.5	-10.6	2.4
XVII-B-382	12.6	38.9	14.3	3.2	20.2	-12.8	64.1	1.1	-9.2	3.6
XVII-B-385	19.6	46.3	16.9	3.2	19.1	-12.6	68.7	0.5	-10.9	1.7
XVII-B-387	9.6	20.6	7.5	3.2	19.1	-12.2	68.7	1.0	-9.3	2.9
XVII-B-388	19.3	39.1	14.3	3.2	18.6	-13.3	58.1	0.9	-10.5	2.8
XVII-B-406	17.1	38.6	14.4	3.1	18.3	-13.2	51.6	0.9	-10.6	2.6
XVII-B-409	16.3	40.5	14.9	3.2	18.0	-12.3	54.8	1.1	-10.0	2.3
XVII-B-410	16.4	37.8	14.0	3.2	19.2	-12.1	50.7	0.4	-9.7	2.4
XVII-B-411	18.5	32.5	12.0	3.2	19.9	-12.8	59.3	1.1	-9.3	3.5
XVII-B-412	18.3	39.5	14.6	3.2	18.5	-12.8	52.5	0.9	-11.1	1.8
XVII-B-446	18.8	39.5	14.6	3.2	19.1	-13.6	50.4	1.0	-11.0	2.6
XVII-B-447	22.7	40.8	15.0	3.2	19.3	-12.9	50.0	1.4	-10.7	2.2
XVII-B-450	7.5	22.6	8.2	3.2	19.5	-12.8	67.1	1.8	-7.5	5.3
XVII-B-453	19.9	34.3	12.6	3.2	19.0	-13.3	56.2	1.1	-10.0	3.3
XVII-B-455	19.6	39.7	14.6	3.2	20.4	-12.2	61.9	1.0	-9.5	2.7
XVII-B-458	18.6	37.9	14.0	3.2	18.9	-12.8	54.7	1.1	-9.8	2.9
XVII-B-459	13.5	40.2	14.8	3.2	19.9	-12.8	52.5	2.2	-10.3	2.5
XVII-B-461	21.4	40.2	14.6	3.2	19.8	-12.7	55.9	0.8	-10.4	2.3
XVII-B-463	22.0	41.2	15.2	3.2	20.5	-13.1	54.0	0.9	-10.6	2.5
XVII-B-473	19.2	37.9	13.8	3.2	19.7	-13.1	51.3	1.1	-10.3	2.8
XVII-B-474	11.8	37.0	13.7	3.2	19.9	-12.8	61.8	1.2	-9.3	3.6
XVII-B-475	20.7	37.7	13.8	3.2	18.7	-13.3	57.7	2.1	-10.7	2.5
XVII-B-476	13.1	39.4	14.5	3.2	19.2	-13.4	59.1	1.2	-10.3	3.2
XVII-B-489	20.9	34.1	12.5	3.2	19.7	-12.6	57.0	2.0	-10.5	2.1
XVII-B-495	21.5	41.5	15.2	3.2	18.8	-13.4	47.3	1.5	-11.4	2.0
XVII-B-496	15.5	38.1	14.0	3.2	19.2	-12.6	60.3	1.2	-8.6	4.0
XVII-B-501	20.2	39.0	14.4	3.2	19.0	-13.0	52.5	0.8	-10.7	2.3
XVII-B-502	20.8	35.8	13.2	3.2	19.8	-13.1	48.7	1.1	-10.7	2.4
XVII-B-504	17.5	50.5	18.6	3.2	19.8	-12.6	50.6	1.0	-10.2	2.4
XVII-B-520	11.0	28.5	10.4	3.2	19.7	-13.6	60.7	1.1	-10.2	3.4
XVII-B-525	17.2	38.6	14.3	3.2	18.6	-13.0	52.0	2.0	-10.6	2.4
XVII-B-886	8.6	23.8	8.7	3.2	19.4	-12.6	66.3	0.8	-8.9	3.7
XVII-B-889	19.0	40.6	14.9	3.2	19.2	-13.4	44.4	0.9	-10.6	2.8
XVII-B-892	22.9	38.5	14.1	3.2	19.5	-12.9	53.5	0.9	-10.8	2.2
XVII-B-893	7.9	27.0	9.8	3.2	18.6	-13.9	63.2	1.2	-9.8	4.1

Together, the results suggest the isotope values generated in this study are authentic, and valid for paleodiet reconstruction.

Analysis of dental collagen provided $\delta^{15}N$ and $\delta^{13}C$ values representing the dietary protein consumed by each Ancestor (Ambrose and Norr 1993). The $\delta^{15}N$ values obtained from collagen ranged from 18.02‰ to 20.53‰ across all Ancestors. Pooled t-tests indicate no significant difference in the mean collagen $\delta^{15}N$ value by site ($p=0.151$), sex ($p=0.479$), labret wear ($p=0.949$), or time period ($p=0.211$). The $\delta^{13}C$ values obtained from collagen ranged from -13.9‰ to -12.0‰ across all individuals sampled. Pooled t-tests indicate no significant difference in the mean collagen $\delta^{13}C$ value by site ($p=0.290$), sex ($p=0.274$), labret wear ($p=0.251$), or time period ($p=0.629$). However, an F-test of variance indicates there is a significant difference ($p=0.013$) in $\delta^{13}C$ values within the labret wear groupings, where individuals with evidence of labret wear exhibit lower within-group variation than those Ancestors without labrets (Figure 2.3).

Analysis of dental apatite provided $\delta^{13}C$ values representing the whole diet consumed by each Ancestor (Ambrose and Norr 1993). Overall, the $\delta^{13}C$ values obtained from apatite ranged from -7.5‰ to -11.4‰ across all Ancestors. Pooled t-tests indicate no significant difference in the mean apatite $\delta^{13}C$ value by site ($p=0.723$) or sex ($p=0.980$). However, a Welch ANOVA test demonstrates the difference in mean $\delta^{13}C$ values between individuals with evidence of labret wear (-10.5‰) and individuals without (-10.0‰) is significant ($p=0.049$) (Figure 2.4). The difference in mean $\delta^{13}C$ values between Ancestors from the Middle Pacific period (-9.9‰) and Late Pacific period (-10.4‰) is also significant ($p=0.040$) (Figure 2.5).

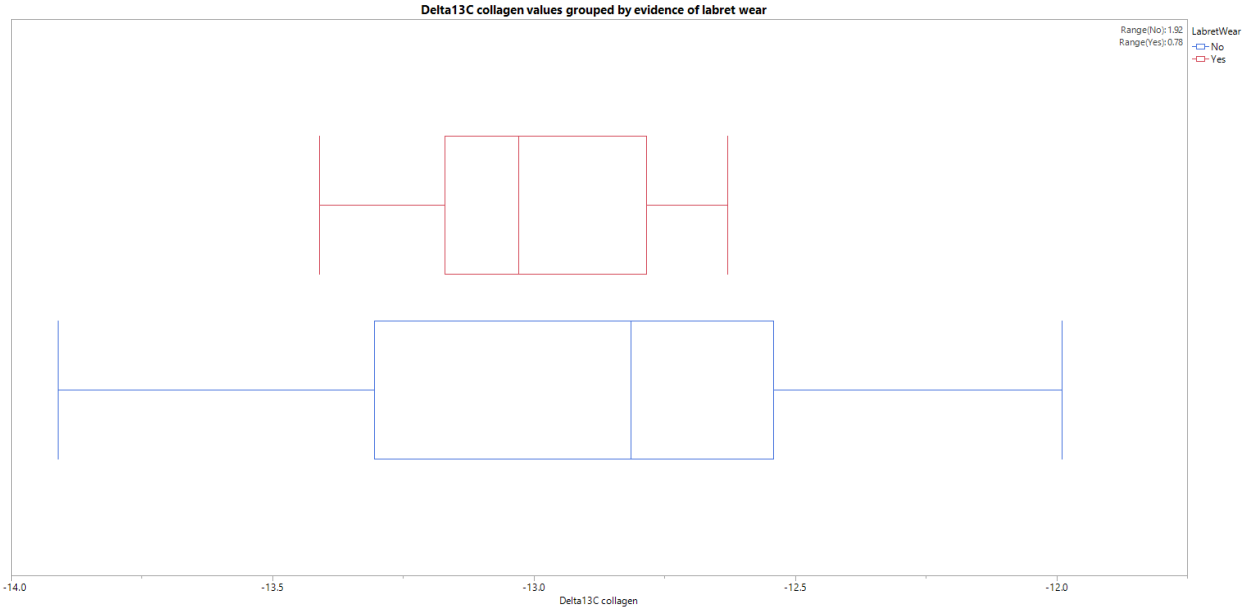

Figure 2.3. Range of $\delta^{13}C$ values obtained from collagen purified from tooth roots. Individuals are grouped by evidence of labret wear, inferred to represent status within the Ancestral Coast Tsimshian community.

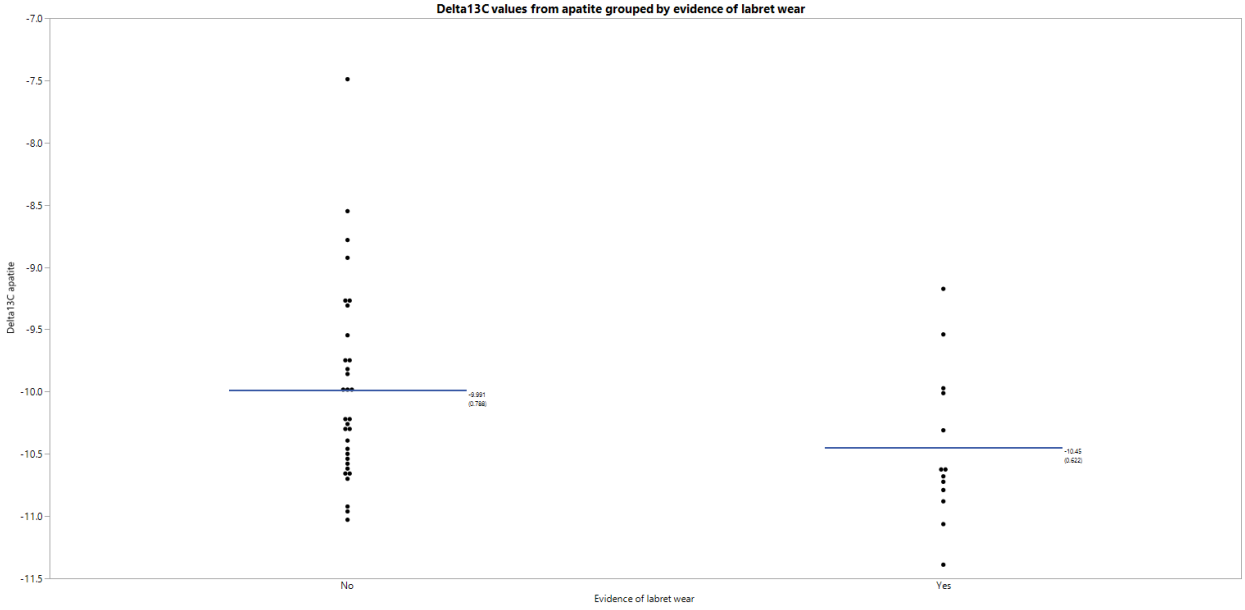

Figure 2.4. Mean $\delta^{13}C$ values derived from the tooth root apatite of Ancestors with evidence of labret wear, inferred to be higher status, and without.

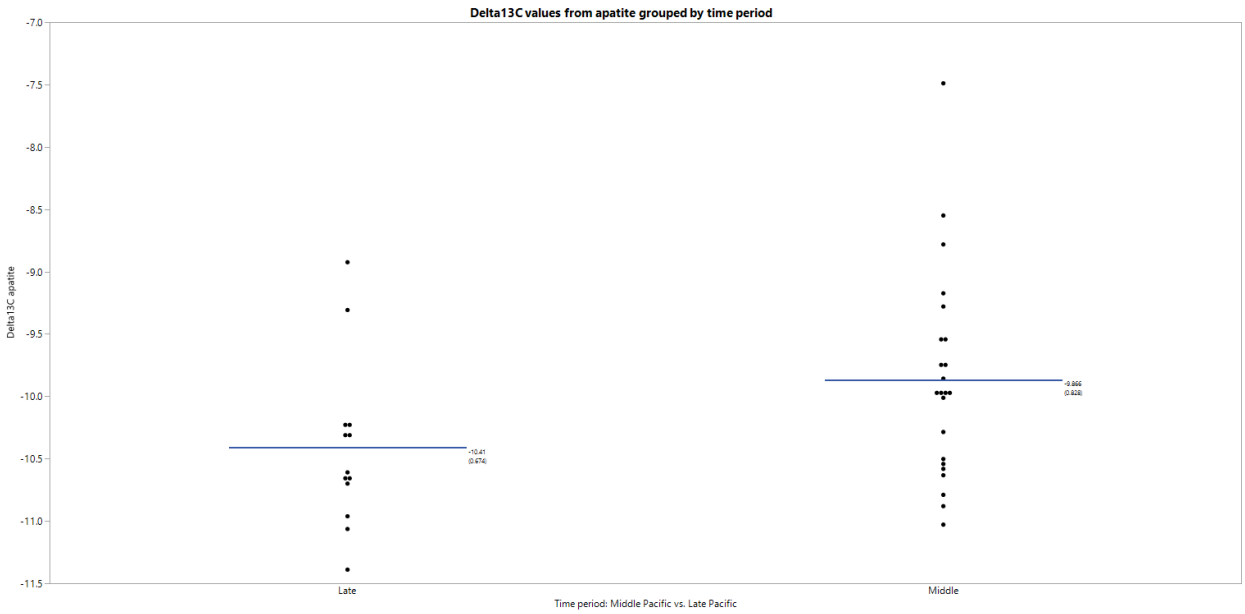

Figure 2.5. Mean δ13C values derived from tooth root apatite. Individuals interred during the Middle Pacific period (3500-1500BP) and Late Pacific (1500-500BP) periods are compared.

A linear regression with t-test was used to examine how well the stable isotope values from dental collagen and apatite explain the dental pathologies observed within the community of Ancestors. The $\delta^{13}C$ values obtained from collagen and apatite do not have a significant relationship with the percent of abscessed alveoli observed in Ancestors (collagen p=0.577, apatite p=0.139). However, $\delta^{15}N$ does have a significant inverse relationship to the percent of abscessed alveoli (p=0.041), where individuals with higher $\delta^{15}N$ have a lower proportion of abscesses.

The same test indicates $\delta^{13}C$ values obtained from collagen have a significant positive relationship with the proportion of AMTL observed in Ancestors (p=0.038), where individuals with less negative $\delta^{13}C$ values have a higher proportion of AMTL. The $\delta^{13}C$ values from apatite

and $\delta^{15}N$ values from collagen do not have a significant relationship with the observed proportion of AMTL (apatite p=0.537, collagen p=0.274).

The relationship between periodontitis and diet was examined using a logistic regression with a chi-square test. The $\delta^{13}C$ and $\delta^{15}N$ values obtained from collagen do not explain the presence of periodontitis within the community of Ancestors ($\delta^{13}C$ p=0.655, $\delta^{15}N$ p=0.324), nor do they have a significant relationship with the severity of periodontitis observed ($\delta^{13}C$ p=0.655, $\delta^{15}N$ p=0.742). The $\delta^{13}C$ value obtained from apatite also lacks a significant relationship to periodontitis presence (p=0.992) and severity (p=0.896).

Similarly, a logistic regression was used to examine how well the stable isotope values explain the cranial lesions observed. The apatite $\delta^{13}C$ value does not have a significant relationship to cribra orbitalia (p=0.431) or porotic hyperostosis (p=0.166) prevalence amongst Ancestors. Neither the $\delta^{13}C$ or $\delta^{15}N$ values from collagen have a significant relationship with the prevalence of cribra orbitalia ($\delta^{13}C$ p=0.145, $\delta^{15}N$ p=0.818) or porotic hyperostosis ($\delta^{13}C$ p=0.312, $\delta^{15}N$ p=0.520).

Genomic analyses

Ancient DNA extracted from the dental calculus (calcified dental plaque) of each Ancestor was sequenced and analyzed for genomic material from possible dietary resources. DNA was successfully extracted and sequenced from the dental calculus of all 45 Ancestors. After quality filtering and inspection (described in detail in *Methods*), a total of 4 different eukaryotic species have been tentatively identified as dietary contributions (see Table 2.3).

Table 2.3. Possible dietary contributions identified via genomic analyses of calculus			
Species	**Common name**	**# Sequences**	**Source library**
Oeamnos americanus	Rocky Mountain goat	1	XVII-B-496 (Ancestor)
Oncorhynchus kisutch	Coho/silver salmon	2	XVII-B-347 (Ancestor)
		1	BC4610 (soil sample)
Oncorhynchus mykiss	Rainbow trout, steelhead	5	XVIIB-347 (Ancestor)
		3	XVII-B-347 rep. (Ancestor)
		2	BC4610 (soil sample)
		1	XVII-B-496 (Ancestor)
Oncorhynchus tshawytscha	Chinook/king salmon	2	XVII-B-347 (Ancestor)
		2	XVII-B-347 rep. (Ancestor)

Discussion

The results of the paleodiet reconstruction within the ancestral Coast Tsimshian community support previous faunal and isotopic analyses suggesting marine resources are a critical component of Coast Tsimshian diet (Chisholm et al. 1983; Coupland et al. 2010; Stewart and Stewart 1996). However, the combination of collagen and apatite analyses, and attention to dietary variation in relation to individual social variables, provides valuable new insight into increasing social complexity during the Middle and Late Pacific periods.

Overall, the stable isotope values obtained from the Coast Tsimshian Ancestors illustrate a high-protein, marine-focused diet (Figure 2.6). The range of $\delta^{13}C$ values obtained from the ancestors are illustrative of the high marine dietary composition, as apatite from an individual with an exclusively terrestrial diet would generate $\delta^{13}C$ values of approximately -26‰ (Ambrose 1993). These values are consistent with the central role of marine resources in Coast Tsimshian culture. The potlatch ceremony, a hallmark of Northwest Coast culture, is crucial to defining and maintaining the highly stratified social order of Coast Tsimshian society (Kan 1986; Piddocke 1965). Feasting is a central component of these gatherings, during which huge volumes of marine mammal meat, as well as fish and fish oil are both consumed and redistributed as signs of

wealth (Kan 1986; Piddocke 1965). The high $\delta^{15}N$ values and tight range suggest the entire community is consuming predominantly high trophic level protein, such as marine mammals and higher trophic fish, like salmon. Consuming marine mammals results in a $\delta^{15}N$ value in the range of 19-21‰, while marine fish results in a range of approximately 18-20‰ (Schwarcz et al. 2014). Some individuals, who exhibit lower $\delta^{15}N$ values, may have been consuming a greater proportion of low trophic marine resources, such mollusks.

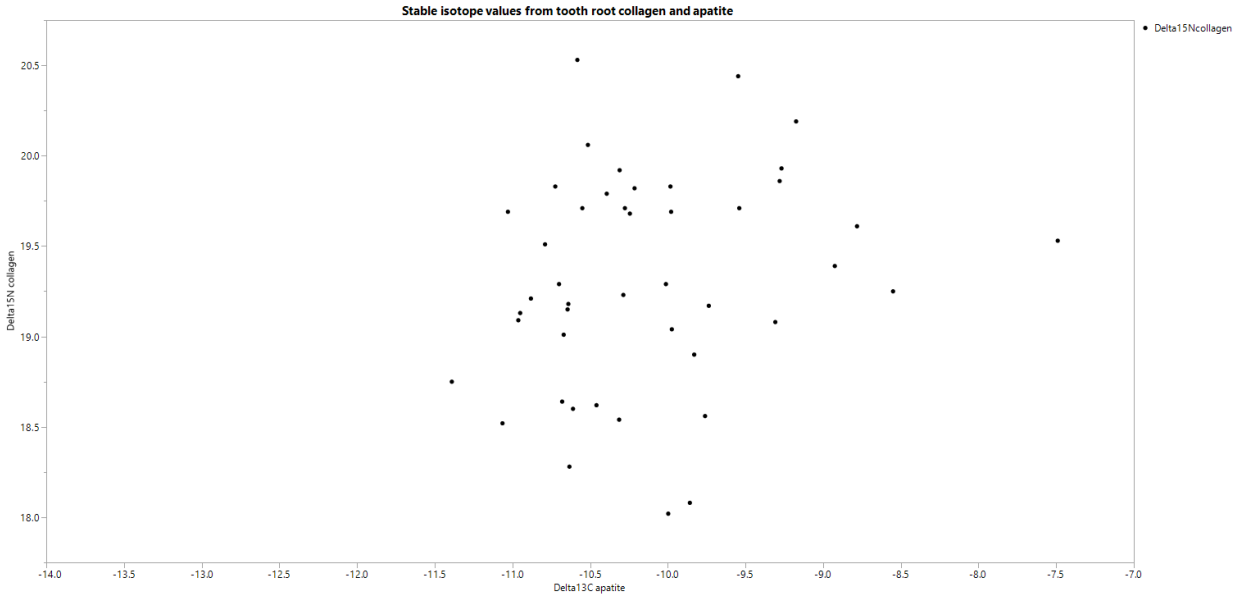

Figure 2.6. Representation of ancestral Coast Tsimshian diet using $\delta^{15}N$ values to illustrate trophic level of dietary protein and $\delta^{13}C$ values from apatite to illustrate marine versus terrestrial contributions to whole diet.

The $\delta^{13}C$ values obtained from both dental collagen and apatite provide a means to estimate the proportion of marine resource contribution to the ancestral Coast Tsimshian diet. By comparing the $\delta^{13}C$ values obtained from the Ancestors' dental tissue with the known $\delta^{13}C$ value for C3 terrestrial plants and the tissue fractional factor (difference between the isotopic value of

the dietary resource and the consumer's tissue value), it is possible to estimate the proportion of marine resource in the dietary protein (Equation 1) and the individuals' whole diet (Equation 2).

Equation 1. Percent of marine resources in diet protein, modified from Ambrose (1993)

$$\%\ marine\ diet = \frac{-26.5\ -\ \delta^{13}C_{collagen} - 5.1}{11} \times -100$$

where -26.5 is the $\delta^{13}C$ value of the C3 plant end member, -5.1 is the tissue fractionation factor for collagen, and 11 is equal to the average spacing between marine and terrestrial resource isotope values estimated for the Northwest Coast food web.

Equation 2. Percent of marine resources in whole diet, modified from Ambrose (1993)

$$\%\ marine\ diet = \frac{-26.5\ -\ \delta^{13}C_{apatite} - 9.4}{11} \times -100$$

where -26.5 is the $\delta^{13}C$ value of the C3 plant end member, -9.4 is the tissue fractionation factor for apatite, and 11 is equal to the average spacing between marine and terrestrial resource isotope values estimated for the Northwest Coast food web.

The equations indicate that Ancestors are obtaining 75-94% of their dietary protein from marine sources rather than terrestrial or riverine environments (Figure 2.7). Analysis of the whole diet indicates 56-95% of the diet is originating from marine resources, suggesting some of the Ancestors are consuming a large proportion of non-marine sources, likely non-proteins. Chisholm et al. (1983) and later Schwarcz et al. (2014) analyzed bone collagen from Ancestors along the Northwest Coast, including the Prince Rupert Harbour area and estimated, similar to the results presented here, marine resources contributed >80% of dietary protein. However, these authors did not analyze apatite, thus their dietary reconstruction did not examine the whole diet of Coast Tsimshian Ancestors. Ambrose and Norr (1993) illustrated that in populations with a high protein diet, like the ancestral Coast Tsimshian, the contribution of dietary carbohydrates

are underrepresented in isotope values derived from collagen. Thus it is critical to analyze both collagen and apatite to fully reconstruct paleodiet in this population.

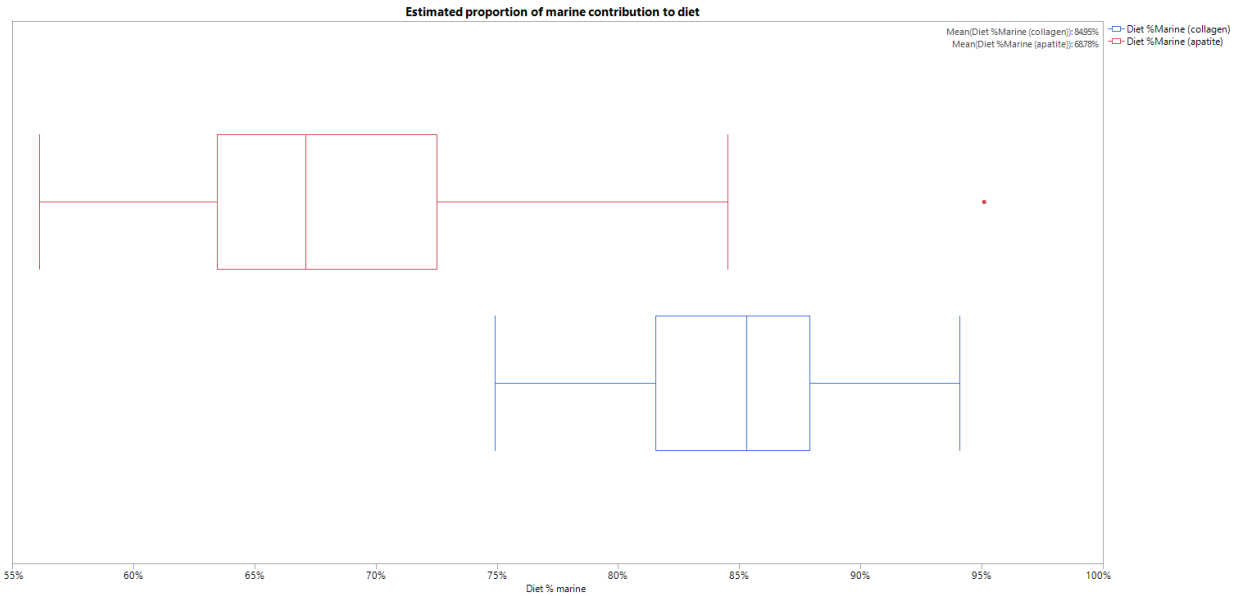

Figure 2.7. Different estimates of marine-resource contribution to diet based on collagen isotopes, reflecting dietary protein, and apatite isotopes, reflecting whole diet.

Individuals with evidence of labret wear exhibit less inter-individual variation in collagen $\delta^{13}C$, indicating their sources of dietary protein are more similar to each other than individuals without labret wear (Figure 2.3). This suggests there may be a core "menu" of high status foods which other community members were not necessarily excluded from eating, but may have had less frequent access to. Thus individuals without labret wear may have expanded their menu in variable ways. Alternatively, wearing these large lip plugs may have made some types of food more challenging to eat, resulting in reduced dietary breadth amongst higher status individuals (Metlakatla pers. comm. 2019). Individuals exhibiting evidence of labret wear also have more negative $\delta^{13}C$ values derived from apatite, representing their whole diet (Figure 2.4). Fat

consumption is not well represented by collagen isotope values but can be discerned from apatite isotope values (Ambrose and Norr 1993). Since lipids are ^{13}C-depleted compared to carbohydrates and protein (DeNiro and Epstein 1978), the more negative $\delta^{13}C$ values observed amongst Ancestors with evidence of labret wear could indicate these individuals were consuming more dietary fat. One possible explanation is increased consumption of eulachon grease. Eulachon (or ooligan, *Thaleichthys pacificus*) is a fish of economic and cultural importance to the Coast Tsimshian. The small, greasy smelt were rendered into grease and exchanged through exchange networks throughout the Northwest Coast (Beynon 1980; Patton and Orchard 2017; Kuhnlein and Chan 1998). Alternatively, Ancestors may have been preferentially consuming higher fat portions of the same dietary protein consumed by the entire community. Descendant community members suggest these high fat sources could be salmon bellies or eyes (Metlakatla pers. comm. 2019). A study of the lipid content of by-products of commercial fish processing reports in yellowfin tuna, for example, fish eyeballs and belly meat were comprised of 4.9% and 1.1% lipids, compared to the 0.5% lipids in white meat portions of the fish. The fish heads and red meat contained the highest total lipid content (7.9% and 7.2%, respectively) (Panggat and Shindo 2002).

There was a significant shift in the whole diet of Ancestors, measured by apatite $\delta^{13}C$ values, between the Middle Pacific period and Late Pacific period (Figure 2.5). Ancestors living during the Late Pacific period have more negative $\delta^{13}C$ values, associated with more terrestrial dietary components or higher fat consumption. Since there was not a corresponding shift in the isotopic values from collagen, the more negative $\delta^{13}C$ values could represent increased exploitation of terrestrial non-protein sources, like berries and other low-protein plants. In response to increasing warfare during the Middle Pacific period, the Coast Tsimshian retreated

from the coast for several hundred years between the Middle and Late Pacific periods, seeking refuge with other Tsimshian villages up the Skeena (Archer 2001; Martindale and Marsden 2003). The Coast Tsimshian could have brought with them a new preference for, or habit of, exploiting terrestrial non-protein foods upon returning to the coast at the start of the Late Pacific period. Alternatively, the shift to more negative $\delta^{13}C$ values could again indicate increased consumption of fat. Ooligan grease was used as a food preservative, and there is archaeological evidence of increased labor devoted to intensive salmon processing and storage (Ames 1994; Ames 2001).

Diet does drive aspects of dental health across the total sample of Coast Tsimshian Ancestors. Higher $\delta^{15}N$ values, indicative of consuming dietary protein from higher trophic levels, was significantly correlated with lower rates of alveolar abscesses. As one possible explanation for this inverse relationship, consuming higher trophic proteins, rather than shellfish, could reduce damage to the dentition and alveolus from abrasion, which could contribute to the development of abscesses. Additionally, less negative $\delta^{13}C$ values from collagen, indicative of increased consumption of dietary protein from marine sources, were significantly correlated with higher proportions of AMTL. Antemortem tooth loss can be caused by a number of factors, including infection and/or regression of the alveolus, tooth damage or disintegration from infection or extreme wear, or other trauma to the mouth, such as a facial impact during interpersonal conflict. Similar to the suggested relationship between higher $\delta^{15}N$ values and reduced abscess rates, the increased consumption of marine protein indicated by less negative $\delta^{13}C$ values from collagen could cause higher AMTL as a result of abrasion from these marine food sources. Alternatively, these data could be correlated through a lifestyle factor not visible in the data collected. For example, community members responsible for fishing, marine mammal

hunting, or raiding/warfare may consume more marine protein and have higher incidences of facial trauma contributing to AMTL.

The hypothesized relationship between social stratification, inter-individual dietary variation, and osteological indicators of health inequality is supported by the data observed in the Coast Tsimshian Ancestors. Dietary isotope values derived from dental apatite, reported from these sites for the first time, reveal dietary variation driven by status and changes in diet possibly related to intensification of food resource processing and storage corresponding to emergence of non-egalitarian social complexity. While there were variations in oral health related to diet, there was no direct relationship between skeletal and dental evidence of health inequality and the indicators of social stratification. As a whole, these data presents a new perspective toward understanding the individual-level impact of increasing social complexity within the ancestral Coast Tsimshian community during the Middle and Late Pacific periods.

Materials and methods

Sample collection

All Ancestors included in this study were previously excavated from the Boardwalk (GbTo-31) and Lachane (GbTo-33) sites as part of the Prince Rupert Harbour Archaeological Project. Their physical remains are currently housed at the Canadian Museum of History in Gatineau, Québec, Canada. The collection of individuals was first surveyed to identify Ancestors with adequate dental calculus deposits. Amongst these Ancestors, individuals were selected to represent both excavation/village sites, males and females (based on documentation from previous analyses completed by CMH researchers), multiple time periods (based on documentation and unpublished carbon dating data provided by Cybulski), and individuals with

and without evidence of labret wear (Cybulski 2015). Individuals had to have third molars fully erupted (or evidence of complete root development in alveolus) to be included in the study sample.

Ancestors selected for inclusion in the study were macroscopically analyzed at the Canadian Museum of History, primarily focusing on the crania. Sex was estimated based on cranial morphology. While sex was recorded following the standards of Buikstra and Ubelaker (1994), the individuals classified as "probable" male or female were merged with the "definite" male or female categories to strengthen statistical analyses of the category. Minimum age was estimated via dental occlusal wear (Lovejoy 1985).

Following the standards established by Buikstra and Ubelaker (1994), dentition presence and development was inventoried and data on dental lesions collected. Antemortem tooth loss is reported as the percentage of healed alveolar sockets out of total observable sockets. Carious lesions were coded according to location on the tooth and the proportion of carious lesions is presented as the percentage of observable teeth in the mouth. Location of oral abscesses was noted and the percent of abscessed sockets out of total observable, non-resorbed sockets reported. Calculus location and severity was noted for each tooth.

The coding scheme developed by the Global History of Health Project (Steckel et al. 2011) was used to record osteological evidence of periodontitis, linear enamel hypoplasias, anemia, and cranial trauma. Severity of periodontitis was scored according to the shape of the alveolar margin along the mandibular left second molar. Enamel hypoplasia was recorded for the left maxillary and mandibular central incisors and canines, scored by severity. Cribra orbitalia and porotic hyperostosis presence and severity were recorded. Pathological changes at the temporomandibular joint were noted. Possible cranial trauma (nasal, other facial, and vault) was

described as antemortem or perimortem based on evidence of healing, with the lesion shape and the maximum diameter recorded.

In addition to osteological analyses, physical samples were collected from each Ancestor and transported to the University of Illinois for destructive analyses. A third molar, preferentially one with dental calculus adhered, was extracted from each individual for isotopic analysis of the tooth root. However, a third molar could not be obtained from individuals 339 and 455, so a first molar and second molar (respectively) were extracted. From Ancestors with dental calculus adhered to the extracted tooth root, an additional sample of dental calculus, greater than 0.2g, was removed from the surface of in-situ teeth using a dental scraper and stored in 1.5mL tubes for future isotopic analyses. The adhered dental calculus was used for genomic analyses. From Ancestors without adhered calculus on the extracted molar, 1-2 dental calculus samples were collected for genomic and, if adequate calculus was present, isotopic analyses.

Each tooth was photographed (occlusal surface and all four sides) before destructive analyses. Morphometric data and non-metric traits were also recorded following the standards outlined by Buikstra and Ubelaker (1994).

Isotopic purification and analysis

One third maxillary or mandibular molar (M3) from each individual was selected for isotopic analysis, with the exception of two individuals where a first or second molar was substituted. As dentine, in contrast to bone, does not remodel over an individual's lifetime (Salazar-García et al. 2014), only the root of each tooth, defined as the area below the cemento-enamel junction, was used for isotopic analysis. This microsampling technique (Balasse et al. 2001; Burt and Garvie-Lok 2013) targets only those isotopes which are incorporated into the

tissue from ages 14.5-23.5 years as the third molar roots are developing (AlQahtani et al. 2010). Sampling only this tissue provides paleodietary estimates for the last stage of dental development, which is most likely to overlap with culturally prescribed "adulthood" and not be confounded by isotopes acquired during breastfeeding, weaning, or early childhood (Burt and Garvie-Lok 2013).

Tooth roots were prepared for isotopic analysis in the University of Illinois Environmental Isotope Paleobiogeochemistry Lab (EIP) following standard lab protocols for the extraction and purification of collagen (Ambrose 1990; Hu et al. 2006) and apatite (Balasse et al. 2002). To prevent contamination, the surface of each tooth root (defined in this case as the area below the cemento-enamel junction) was cleaned by removing a thin exterior layer of dentine using a diamond bur. The whole tooth was then sonicated in distilled water and thoroughly dried in a freeze-dryer. After cleaning, the crown was separated from the root at the cemento-enamel junction using a carbide rotary device. The crown was preserved for future macroscopic analyses and/or repatriation. The root was crushed in a mortar and pestle, then sieved and separated into three groups based on grain size: <0.25mm; >0.25mm - <0.5mm; >0.5mm - <1.0mm. All crushed root was retained for analysis.

The fine –grained (<0.25mm) ground tooth root was treated using a 50% Clorox solution to remove possible organic contaminates. Samples were suspended in the Clorox solution overnight, then decanted and rinsed thoroughly with distilled water. Samples were then suspended in 0.1M acetic acid for 2-3 hours to remove absorbed carbonates, before being decanted and rinsed repeatedly with distilled water. To dry, the samples were frozen in a conventional freezer and then transferred to a freeze drier. Apatite carbonate analysis, which provides one measure of $\delta^{13}C$, was completed in the Illinois State Geological Survey Stable

Isotope Lab at UIUC using a Finnegan MAT Kiel III automated carbonate reaction device connected to a Finnigan MAT-252 isotope ratio mass spectrometer.

Collagen was extracted from large-grain (>0.5mm - <1.0mm) crushed tooth root. The samples were demineralized over the course of two days using 0.2M HCl. Samples were then rinsed to pH neutrality using distilled water and treated with 0.125M NaOH overnight. After thoroughly rinsing in distilled water, 10^{-3}M HCl was added to the samples, which were then transferred to a gravity oven set at low temperature (7-75*C) to dissolve the collagen over a period of 24 hours. The dissolved collagen solution was drained through a filter and then condensed in the gravity oven for an additional 24 hours (until approximately 2ml of solution remained), before being drained into a scintillation vial. The condensed collagen solution was then frozen in a conventional freezer before being transferred to the freeze drier. The dried collagen was transported to the Illinois State Geological Survey Stable Isotope Lab for isotopic analysis using a Carlo-Erba NC 2500 elemental analyzer connected to a Thermo Finnigan Conflo IV universal continuous flow interface and Thermo Finnigan Delta V Advantage isotope ratio mass spectrometer. Analysis of the dentine collagen provided measures of $\delta^{13}C$ and $\delta^{15}N$. All three isotope values were obtained for each of the 45 Ancestors analyzed.

DNA extraction and sequencing

All pre-amplification genomic labwork was performed in a dedicated ancient DNA laboratory with appropriate equipment and protocols to minimize environmental contamination of the dental calculus samples. The samples, consisting of either adhered or loose dental calculus, were collected at the Canadian Museum of History and exported to the Malhi Molecular Anthropology Lab at the University of Illinois at Urbana-Champaign for DNA

extraction. Loose dental calculus samples were exposed to ultraviolet light for 20 minutes (per side if the sample was large flakes rather than powder) via a crosslinker to prevent contaminating exogenous DNA from amplifying. Adhered calculus samples were soaked in reagent-grade bleach for three minutes, rinsed three times with molecular-grade water, rinsed once in isopropanol, and dried under ultraviolet light for 20 minutes (each side) before being removed from the tooth using a stainless steel dental scaler. All dental calculus samples were weighed before digestion.

The calculus samples were crushed and then digested for 24-48 hours in a solution of EDTA, proteinase K, and N-lauryl sarcosine at 37 degrees Celsius. After digestion, the ancient DNA in solution was concentrated and then extracted using the Qiagen MinElute PCR Purification kit. The extract was then quantified using a Qubit Fluorometer. For each extraction group, one extraction blank control (EBC/negative) made of crosslinked water was given identical treatment, from digestion through sequencing.

Additionally, two soil samples and an accompanying negative were digested and extracted using the Qiagen Stool Mini kit. The extract was purified twice with the Zymo OneStep PCR Inhibitor Removal kit and inhibitor removal confirmed with a spiked amplification before proceeding to the library build.

A genomic library was built from each extracted DNA sample using the NuGEN Ovation Ultralow Library Prep kit with Illumina-compatible unique dual indexes. Each extract was diluted to 100ng or less of DNA per library build. An additional reagent-only library was also constructed as a final negative control. The libraries were amplified with Phusion HS II for 7-15 cycles, depending on the quantity of DNA in the starting extract. All negative libraries were amplified for 15 cycles.

The amplified libraries were purified using an Agentcourt AMPure bead clean-up. The purified libraries were confirmed via gel electrophoresis and the DNA concentration quantified before being quality checked on an AATI Fragment Analyzer at the Roy J. Carver Biotechnology Center at UIUC. A final size selection was performed prior to sequencing. Each library was sequenced on either the Illumina HISEQ 4000 or NOVASEQ 6000, producing 150bp single-end reads.

Bioinformatic analyses

The sequencing produced 106-8 million unique DNA sequences per dental calculus sample. The sequences were quality trimmed, the adapter removed, filtered to a minimum length of 50bp, and deduplicated before downstream analysis. The sequences were then assembled using ABySS (Simpson et al. 2009), with a kmer of 40. The assembled ancient DNA sequences were matched to reference genomes in the NCBI nucleotide database using BLASTn (Camacho et al. 2009). The taxa identified were then filtered to a minimum query sequence length of 95bp and a 100% identity match to the reference genome of the identified taxon following the recommendations of Warinner et al. (2014). The resulting taxa were imported to MEGAN (Huson et al. 2016) for review.

Taxa which were identified across two or more individuals, had a higher number of sequences in the calculus libraries than extraction blank or negative libraries, and are species native to North America were identified as possible dietary contributions. In MEGAN, the full list of taxonomic matches to the individual sequences from the identified species was inspected. A high proportion of reference genomes in the NCBI nucleotide database are likely contaminated with bacterial genomic material, meaning these species may be identified as matches to

sequences in the dental calculus, but actually be hits to bacteria in the sample, rather than dietary components. One possible dietary contribution, *Strongylocentrus purpuratus* (Pacific purple sea urchin), was excluded because the sequences cross-matched with bacterial species.

CHAPTER 3: CHARACTERIZING THE ORAL MICROBIOME OF AN ANCESTRAL COAST TSIMSHIAN COMMUNITY

Abstract

The human oral microbiome mediates the relationship between diet and oral health, and has the capacity to impact the health of other systems throughout the human body. This chapter examines how the inter-individual diversity in diet and oral health demonstrated to be associated with the increasing social complexity and cultural changes during the Middle and Late Pacific periods is mirrored in the taxonomic composition of the ancestral Coast Tsimshian oral microbiome. The Coast Tsimshian Ancestors have a unique oral microbial composition, dominated by Actinobacteria and Firmicutes, which may be an adaptive response to their high-protein fisher-hunter-gatherer subsistence strategy.

Introduction

The human microbiome has emerged as a rapidly expanding and fruitful area of research, drawing on and connecting to research in human evolution (Davenport et al. 2017; Nasidze et al. 2009; Schnorr et al. 2016; Tito et al. 2012; Warinner et al. 2014; Weyrich 2015) and health (Duran-Pinedo et al. 2015; He et al. 2014; Koren et al. 2010; Scannapieco et al. 2013; Slocum et al. 2016). The oral microbiome is the second largest human-associated microbial community (HMP 2012). As ancient microbial DNA has been shown to preserve extremely well in dental calculus (calcified dental plaque; Warinner et al. 2014), the oral microbiome provides one opportunity to study human evolutionary history.

Dietary intake, including macro- and micro-nutrients, has been demonstrated to impact the taxonomic composition and function of the oral microbiome (Kato et al. 2017; Morgan et al. 2013; Takahashi 2015). Sustained intake of sugar-rich carbohydrates, in particular, has been demonstrated to shift the taxonomic composition of the oral microbiome (Wade 2013). As such, investigating the shift in oral microbial composition in relation to different subsistence diets and lifestyles throughout human evolutionary history is a growing area of research interest (Adler et al. 2013; Nasidze et al. 2011; Ozga et al. 2016; Skelly et al. 2018). Much of this research has focused on the shift from hunter-gatherer subsistence to agriculture, or more recent industrialization, because both of these transitions are hypothesized to involve dramatic, sustained increases in carbohydrate consumption (Adler et al. 2013). Additionally, these studies have used frameworks that compare two separate populations, rather than examining dietary variation within a single population with shared environmental and social contexts. Human oral microbiome research investigating other forms of dietary variation, such as high fat or protein diets, is lacking in comparison to other microbiome research fields, like the gut (Davenport et al. 2017; Kato 2017; Morgan et al. 2013).

The existing field of scholarship has identified key microbial groups associated with health and disease which may be used to interpret the impact of dietary change on individual health experiences in past populations. Periodontitis is a polymicrobial disease and many other bacteria have been found to contribute to the dysbiosis associated with periodontal lesions (Arora et al. 2014; Hajishengallis et al. 2012). The 'red-complex' bacteria, *Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola* are associated with severe periodontitis (Duran-Pinedo and Frias-Lopez 2015; Socransky et al. 1998). Periodontitis infection can not only progress to tooth loss, but also impact systemic health by increasing inflammation correlated

with atherosclerosis (Takahashi et al. 2006; Reyes et al. 2013), rheumatoid arthritis (de Pablo et al. 2008; Joseph et al. 2013), pneumonia (Kimizuka et al. 2003), diabetes (Teng et al. 2002), and cancer (Michaud et al. 2013; Groeger et al. 2011). In contrast, several members of the *Streptococcus* genus, *S. gordonii, S, intermedius, S. mitis, S. oralis,* and *S. sanguis*, form the 'yellow-complex' which, along with 'purple-complex' species *Actinomyces odontolyticus and Veillonella parvula,* are associated with non-infectious sites (Duran-Pinedo and Frias-Lopez 2015). The genera *Actinomyces* and *Lactobacillus,* as well as *Streptococcus mutans* have been documented as the most abundant bacteria in association with carious lesions (Duran-Pinedo and Frias-Lopez 2015; Takahashi and Nyvad 2011).

Importantly, assessments of "healthy" oral microbiomes (HMPC 2012; Zaura et al. 2009; 2014) and studies of disease-associated dysbiosis have not included Indigenous communities, who may have unique microbial taxonomic or functional compositions related to their recent population-specific evolutionary histories and adaptations. Without testing how the microbiome shifts in response to oral health in communities with different evolutionary-adaptive histories, there is a risk of pathologizing microbiomes from non-Western/industrial populations who may have adapted different taxonomic compositions in response to diet or environmental factors. A well-contextualized assessment of the oral microbiome in Indigenous communities is sorely needed to combat existing ascertainment bias (Ozga et al. 2016) in this research field.

While previous studies have explored the composition of the human oral microbiome in relation to hunter-gatherer and agricultural subsistence, this paper examines how the oral microbiome of the ancestral Coast Tsimshian community, as a whole, reflects a fisher-hunter-gatherer subsistence strategy. Additionally, I build on the results of the previous chapter to assess if the inter-individual variation in diet observed between individuals of inferred high and low

status groups, and over time between the Middle Pacific and Late Pacific periods, are mirrored by the taxonomic composition of the oral microbiome.

Results

Metagenomes were successfully sequenced from dental calculus samples collected from each of the 45 Coast Tsimshian Ancestors. Additionally, one soil sample from each excavation site (n=2) was sequenced to control for contamination from the burial environment. Extraction blank controls (EBC/negative libraries) from each set of calculus extractions and library builds (n=6), the soil extraction and library build (n=1) and a library-only reagent control (n=1) were also sequenced to identify possibly microbial contaminants from the laboratory environment, reagents, and plastics. The resulting sequence libraries were subsampled to a depth of 2 million prior to taxonomic classification via nucleotide alignment using MALTn, following previously validated methods (Eisenhofer and Weyrich 2019). The taxonomic classifications were imported into MEGAN6 (Huson et al. 2016), where a series of quality checks were performed prior to downstream analyses. First, the taxonomic composition of the negative libraries was assessed for evidence of cross-contamination from the calculus sample libraries. As there was not evidence of significant oral microbes in the negative libraries, the taxa identified in the negative libraries were subtractively filtered from the dental calculus libraries to ensure downstream analyses were performed on endogenous oral microbes.

Before assessing if any dietary or health variables correlate with microbial diversity, PERMANOVA was used to check if methodological factors like DNA extraction group, tooth position, tooth type, or sequencing platform drove any inter-individual variation in microbial diversity. Prior to subtractive filtering, the extraction group did have a significant influence

(p=0.001) on microbial composition. However, after all dental calculus samples were subtractively filtered, this factor was no longer significant (p=0.299). Tooth type, whether the dental calculus was removed from a molar or multiple positions along the dental arcade, was not a significant variable (p=0.122), nor was the individual tooth position (p=0.750). The metagenomic libraries derived from dental calculus were sequenced using a combination of the Illumina HISEQ 4000 and NOVASEQ 6000 platforms, but sequencing platform did not have a significant impact on Ancestors inter-individual microbial variation (p=0.176).

Alpha diversity

Shannon's index was used to assess the abundance and evenness of microbial taxa within each sample. The results were statistically compared across samples using the Kruskal-Wallis test for pairwise analysis of categorical variables, Spearman correlation for ordinal variables, and Pearson correlation for continuous numeric variables. None of the variables predicted inter-individual differences in oral microbiota species richness and distribution (see Table 3.1).

Beta diversity

Bray-Curtis distance metrics, tested with PERMANOVA (categorical and ordinal variables) and Adonis (continuous variables), were used to identify statistically significant dissimilarity in microbial composition between samples. There was no statistically significant difference in oral microbiome composition between individuals with different dietary isotope compositions, oral and cranial health indicators, or social groupings (see Table 3.2).

Table 3.1. Tests of alpha diversity statistical significance				
Variable	**Statistical test**	***N***	**Groups**	***P* (<0.05)**
Social factors				
Site	Kruskal-Wallis	45	2	0.273
Sex (male/female)	Kruskal-Wallis	45	2	0.892
Status (evidence of labret wear)	Kruskal-Wallis	45	2	0.881
Time period (Middle or Late Pacific)	Kruskal-Wallis	36	2	0.118
Health indicators				
Cribra orbitalia (presence/absence)	Kruskal-Wallis	28	2	0.466
Porotic hyperostosis (presence/absence)	Kruskal-Wallis	35	2	0.792
Periodontitis (presence/absence)	Kruskal-Wallis	30	2	0.561
Periodontitis (severity)	Spearman	30	4	0.880
Caries (presence/absence)	Kruskal-Wallis	44	2	0.348
Caries (percent teeth affected)	Pearson	44	N/A	0.300
Abscesses (presence/absence)	Kruskal-Wallis	44	2	0.445
Abscesses (percent sockets affected)	Pearson	44	N/A	0.374
AMTL (presence/absence)	Kruskal-Wallis	41	2	0.620
AMTL (percent observable)	Pearson	41	N/A	0.282
Dietary isotope values				
$\delta^{13}C$ apatite	Pearson	45	N/A	0.697
Diet percent marine from apatite	Pearson	45	N/A	0.692
$\delta^{13}C$ collagen	Pearson	45	N/A	0.097
$\delta^{15}N$ collagen	Pearson	45	N/A	0.660
Diet percent marine from collagen	Pearson	45	N/A	0.099

Taxa overview

As there was no significant inter-individual variation between the Ancestors' oral microbiome taxonomic composition, their microbiome is discussed at the community, rather than individual, level. The ancestral Coast Tsimshian oral microbiome is dominated by the gram-positive bacterial phyla Actinobacteria (29.4%) and Firmicutes (20.1%), followed by Proteobacteria (16.3%), and Chloroflexi (10.6%) (see Figure 3.1). The phyla Firmicutes, followed by Proteobacteria and Actinobacteria, are represented by the greatest range of microbial genera. The most dominant genera are *Actinomyces* and *Anaerolineaceae* (see Table 3.3).

Table 3.2. Tests of beta diversity statistical significance				
Variable	**Statistical test**	***N***	**Groups**	***P* (<0.05)**
Social factors				
Site	PERMANOVA	45	2	0.690
Sex (male/female)	PERMANOVA	45	2	0.314
Status (evidence of labret wear)	PERMANOVA	45	2	0.415
Time period (Middle or Late Pacific)	PERMANOVA	36	2	0.300
Health indicators				
Cribra orbitalia (presence/absence)	PERMANOVA	28	2	0.407
Porotic hyperostosis (presence/absence)	PERMANOVA	35	2	0.154
Periodontitis (presence/absence)	PERMANOVA	30	2	0.224
Periodontitis (severity)	PERMANOVA	30	4	0.443
Caries (presence/absence)	PERMANOVA	44	2	0.096
Caries (percent teeth affected)	Adonis	44	N/A	0.080
Abscesses (presence/absence)	PERMANOVA	44	2	0.162
Abscesses (percent sockets affected)	Adonis	44	N/A	0.811
AMTL (presence/absence)	PERMANOVA	41	2	0.990
AMTL (percent observable)	Adonis	41	N/A	0.216
Dietary isotope values				
$\delta^{13}C$ apatite	Adonis	45	N/A	0.325
Diet percent marine from apatite	Adonis	45	N/A	0.311
$\delta^{13}C$ collagen	Adonis	45	N/A	0.194
$\delta^{15}N$ collagen	Adonis	45	N/A	0.939
Diet percent marine from collagen	Adonis	45	N/A	0.195

Correlation with oral health

To further assess if the microbial taxa shift in response to oral health within the ancestral community, an ANOVA test was employed to identify significant differences in abundance of individual taxa in relation to the presence or absence of oral pathologies. There are six species from the phyla Actinobacteria and Firmicutes with significant variation in abundance in relation to the presence/absence of carious lesions (see Table 3.4). Two additional taxa from the phyla Bacteroidetes and Firmicutes varied significantly in relation to the presence/absence of periodontitis. There were no taxa with significant variance in abundance in relation to the presence of alveolar abscesses or antemortem tooth loss.

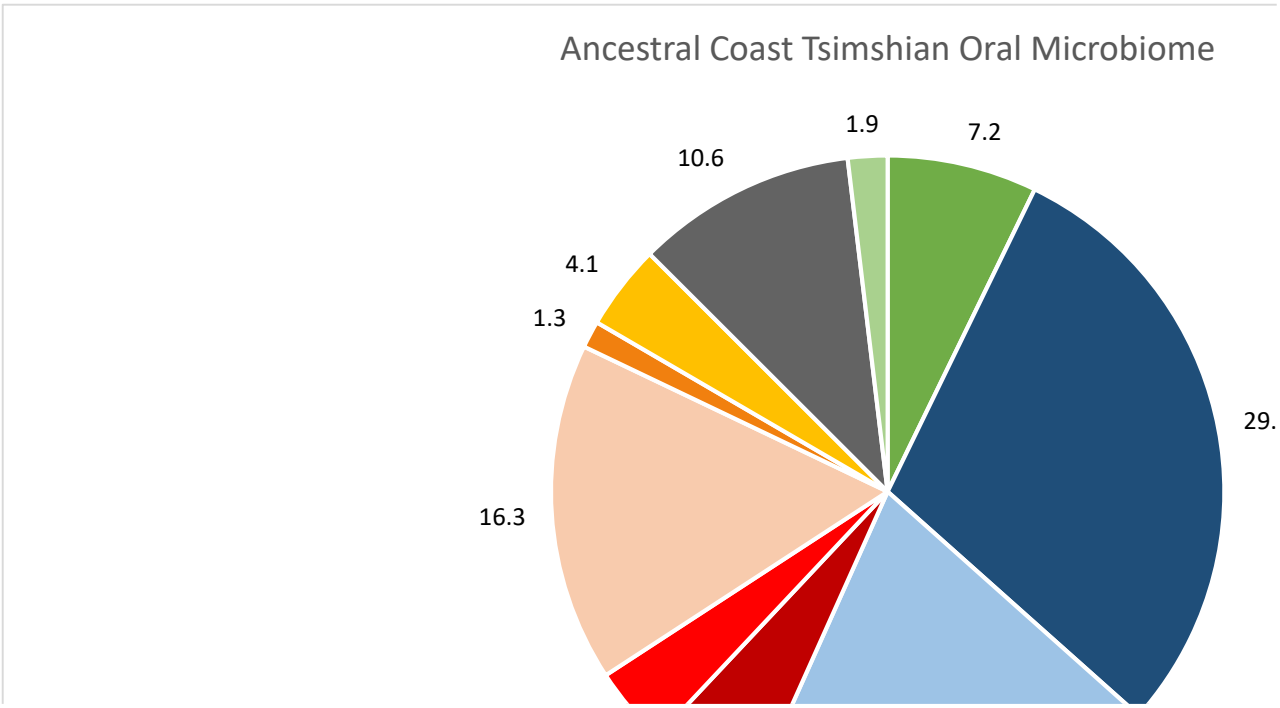

Figure 3.1. Relative abundance of bacterial phyla in ancestral Coast Tsimshian oral microbiome.

Table 3.3. Genera, sorted by phyla, identified in the ancestral Coast Tsimshian oral microbiome at an abundance greater than 0.1%		
Phylum	**Genus**	**Abundance**
Euryarchaeota	*Methanobrevibacter*	7.2%
Actinobacteria	*Actinomyces*	15.3%
	Corynebacterium	1.3%
	Rothia	0.6%
	Propionibacterium	7.4%
	Olsenella	3.7%
Bacteroidetes	*Porphyromonas*	0.4%
	Tannerella	2.1%
	Capnocytophaga	1.4%
Chlorobi	*Chlorobium*	0.2%
Chloroflexi	*Anaerolineaceae*	10.6%
Firmicutes	*Gemella*	1.0%
	Abiotrophia	0.4%
	Streptococcus	3.9%
	Clostridium	0.9%
	Eubacterium	2.0%
	Pseudoramibacter	0.2%
	Catonella	0.3%
	Johnsonella	1.9%
	Lachnoanaerobaculum	0.3%
	Filifactor	0.2%
	Selenomonas	0.8%
	Veillonella	0.3%
	Parvimonas	1.5%
	Peptoniphilus	0.2%
Fusobacteria	*Fusobacterium*	2.2%
	Leptotrichia	1.5%
Proteobacteria	*Lautropia*	4.4%
	Ottowia	3.5%
	Eikenella	0.8%
	Desulfobulbus	0.3%
	Desulfomicrobium	2.3%
	Campylobacter	0.9%
	Cardiobacterium	1.2%
	Aggregatibacter	1.4%
Spirochaetes	*Treponema*	1.2%
	Fretibacterium	3.5%

Table 3.4. Species with significant variation in abundance related to oral pathologies			
Taxa associated with carious lesions	**Mean associated with PRESENCE**	**Mean associated with ABSENCE**	**ANOVA FDR_P**
Actinomyces spp. oral taxon 848	4191.8	362.8	0.002
Propionibacterium freudenreichii	1090.5	0	0.043
Olsenella uli	1323.5	0	0.043
Streptococcus intermedius	968.3	0	0.043
Peptostreptococcus anaerobius	1037.5	0	0.043
Dialister invisus	991.3	0	0.043
Taxa associated with periodontitis	**Mean associated with PRESENCE**	**Mean associated with ABSENCE**	**ANOVA FDR_P**
Phocaeicola abscessus	0	1277.5	0.008
Johnsonella ignava	15550.3	69885.5	0.023

Discussion

Despite the differences in diet observed between Coast Tsimshian Ancestors of different status, inferred from labret wear, and the change in diet over time noted between the Middle Pacific Period and Late Pacific Period, there were no significant differences in oral microbiome composition across the ancestral community. Previous studies identifying significant oral microbial variation using ancient dental calculus have compared populations with dramatically different subsistence strategies, namely comparing hunter-gatherer populations to agriculturalists (Adler et al. 2013, for example). In contrast, this study examines within-population variation, amongst individuals sharing a fisher-hunter-gatherer lifestyle. While there is statistically significant dietary variation identified within the ancestral Coast Tsimshian community, via stable isotope analysis, the differences in means between groups were less than 2‰. This dietary difference may be too slight to drive changes in taxonomic composition within the oral microbiome. It is also possible the oral microbiome does not respond with the same sensitivity to shifts in dietary fat, which these inter-individual variations were correlated with, as it does to influxes of carbohydrates. Additionally, factors like host genome and horizontal and vertical

transmission of microbiota (Davenport et al. 2017) may make it more difficult to identify microbial variation within a community than across populations.

Similarly, there was no significant difference in the ancestral oral microbiome in correlation with the health indicators measured. However, there was a significant shift in mean abundance of some individual taxa in relation to the presence or absence of carious lesions and periodontitis. *Propionibacterium freudenreichii* is a gram-positive bacteria associated with the presence of carious lesions in the ancestral oral microbiome. Is has probiotic properties (Meurman and Stamatova 2007), and contributes to the production of B_{12} (Hashimoto et al. 1996). Its primary metabolic product resulting from the fermentation of glucose is propionic acid (Patrick and McDowell 2015), which may explain why its abundance is positively associated with carious lesions. *Olsenella uli* has previously been noted in ancient dental calculus samples (Warinner et al. 2014) and is associated with carious lesions, endodontic infection, periodontitis and periodontal abscess (Rocas and Siqueira 2005; Wade et al. 2005). *Dialister invisus* has similarly been detected in association with periodontitis and periodontal abscesses (Rocas and Siqueira 2005). Overall, the bacteria associated with carious lesions in the ancestral oral microbiome seem to serve analogous roles to *S. mutans* bacteria in creating and maintaining the acidic environment necessary for carious lesions, and have been found in association with several oral pathologies. In contrast, the bacteria *Phocaeicola abscessus* and *Johnsonella ignava* were found to increase in abundance in the absence of periodontitis, which is unexpected based on the current literature. *Phocaeicola abscessus* has been previously associated with endodontic infection and brain abscesses (Anderson et al. 2012), but is not associated with infection in the ancestral Coast Tsimshian oral microbiome. Similarly, *Johnsonella ignava* is noted in the literature to occur in association with gingivitis and oral cancers (Moore et al. 1994; Pushalkar et

al. 2012), but exhibits reduced abundance in relation to gingivitis in the ancestral Coast Tsimshian oral microbiome. This mismatch between the clinical literature on oral inflammatory-associated bacteria and the observed presence of these taxa in the ancestral Coast Tsimshian oral microbiome suggests there may be ascertainment bias is clinical interpretations of immunostimulatory bacteria. Clinical studies of oral pathologies rarely include Indigenous North American individuals, which may be limiting our conceptualizations of what is "healthy" and "pathogenic" within the oral microbiome. Functional composition analysis is needed illuminate the role of these bacteria in how the ancestral oral microbiome has adapted to their evolutionary history and subsistence.

At the phylum level, the composition of the ancestral microbiome most closely resembles other ancient dental calculus microbiomes sampled from hunter-gatherer populations with high protein diets (Weyrich et al. 2017), and is dramatically different from the calculus microbiomes analyzed from past populations with agricultural subsistence practices (Adler et al. 2013), as well as plaque samples from non-Indigenous industrialized populations. These populations tend to have oral microbiomes dominated by the gram-negative bacterial phyla. In contrast, the oral microbiome of the Coast Tsimshian community is dominated by predominantly gram-positive Actinobacteria and Firmicutes. This suggests the high-protein diet of the Coast Tsimshian has played a role in shaping the evolution of the ancestral oral microbiome. Future analyses focusing on the functional role of the bacteria within the ancestral oral microbiome may reveal specific adaptations to the marine-focused diet. For example, a study of the gut microbiota of Japanese individuals suggests these individuals acquired genes for carbohydrate active enzyme from bacteria associated with seaweed in the daily diet (Hehemann et al. 2010).

In conclusion, the ancient DNA sequenced from the dental calculus of Coast Tsimshian Ancestors has provided a much-needed characterization of the oral microbiome of an ancestral fisher-hunter-gatherer community. While overall diet may have shaped the composition of the ancestral Coast Tsimshian microbiome, inter-individual variation in diet within the community of Ancestors does not appear to drive taxonomic variation in the oral microbiome. Thus the inter-individual differences in diet related to status and social change between the Middle and Late Pacific periods were not mirrored in the oral microbiome taxonomic composition. However, variation in mean abundance of specific taxa was observed in relation to carious lesions and periodontitis. While the bacterial species associated with the presence of carious lesions were analogous to the gram-positive, acid-producing bacteria most commonly associated with carious lesions in the clinical literature, the bacteria associated with periodontitis absence was not consistent with previous clinical description. This highlights a possible ascertainment bias in current clinical understandings of the role of inflammation-associated bacteria in the oral microbiome. Additional research exploring the functional composition of the ancestral oral microbiome is needed to further examine if microbial interactions within the core microbiome of the ancestral community are changing in relation to diet and social change.

Materials and methods

DNA extraction and sequencing

All pre-amplification genomic labwork was performed in a dedicated ancient DNA laboratory with appropriate equipment and protocols to minimize environmental contamination of the dental calculus samples. The samples, consisting of either adhered or loose dental calculus, were collected at the Canadian Museum of History and exported to the Malhi

Molecular Anthropology Lab at the University of Illinois at Urbana-Champaign for DNA extraction. Loose dental calculus samples were exposed to ultraviolet light for 20 minutes (per side if the sample was large flakes rather than powder) via a crosslinker to prevent contaminating exogenous DNA from amplifying. Adhered calculus samples were soaked in reagent-grade bleach for three minutes, rinsed three times with molecular-grade water, rinsed once in isopropanol, and dried under ultraviolet light for 20 minutes (each side) before being removed from the tooth using a stainless steel dental scaler. All dental calculus samples were weighed before digestion.

The calculus samples were crushed and then digested for 24-48 hours in a solution of EDTA, proteinase K, and N-lauryl sarcosine at 37 degrees Celsius. After digestion, the ancient DNA in solution was concentrated and then extracted using the Qiagen MinElute PCR Purification kit. The extract was then quantified using a Qubit Fluorometer. For each extraction group, one extraction blank control (EBC/negative) made of crosslinked water was given identical treatment, from digestion through sequencing.

Additionally, two soil samples and an accompanying negative were digested and extracted using the Qiagen Stool Mini kit. The extract was purified twice with the Zymo OneStep PCR Inhibitor Removal kit and inhibitor removal confirmed with a spiked amplification before proceeding to the library build.

A genomic library was built from each extracted DNA sample using the NuGEN Ovation Ultralow Library Prep kit with Illumina-compatible unique dual indexes. Each extract was diluted to 100ng or less of DNA per library build. An additional reagent-only library was also constructed as a final negative control. The libraries were amplified with Phusion HS II for 7-15

cycles, depending on the quantity of DNA in the starting extract. All negative libraries were amplified for 15 cycles.

The amplified libraries were purified using an Agentcourt AMPure bead clean-up. The purified libraries were confirmed via gel electrophoresis and the DNA concentration quantified before being quality checked on an AATI Fragment Analyzer at the Roy J. Carver Biotechnology Center at UIUC. A final size selection was performed prior to sequencing. Each library was sequenced on either the Illumina HISEQ 4000 or NOVASEQ 6000, producing 150bp single-end reads.

Bioinformatic analyses

The sequencing produced 168-20 million DNA sequences per dental calculus sample. The sequences were quality trimmed, the adapter removed, and filtered to a minimum length of 50bp before taxonomic assignment. Two methods of taxonomic assignment were tested before downstream analysis. The bioBakery metagenomic profiling tool suite (KneadData, MetaPhlAn; McIver et al. 2018) uses marker genes to identify taxa based on alignment, while MALT (Herbig et al. 2016) uses a full-genome alignment approach.

To test taxonomic classification in bioBakery, the sequences were filtered with KneadData against HG19 to remove any human DNA, and against a custom database containing all of the sequenced extraction, library, and soil negative controls to remove potential cross-contamination or reagent-based contaminates. The trimmed and filtered sequence files were then aligned to the microbial marker-gene reference database to obtain taxonomic classification and relative abundance statistics.

For comparison, each set of trimmed sequences was subsampled to two million sequences and aligned to the RefSeqGCS database using MALTn nucleotide alignment (Eisenhofer and Weyrich 2019). The resulting taxonomic classifications were imported into MEGAN6 (Huson et al. 2016) and visually assessed before all extraction, library, and soil controls were subtractively filtered from the taxonomic classification results.

As MALT has been demonstrated to perform better on the short, fragmented DNA sequences characteristic of ancient DNA samples (Weyrich et al. 2017) than other alignment-based classification tools, downstream statistical analyses and visualization of microbial community composition in QIIME (Caporaso et al. 2010) and QIIME2 (Bolyen et al. 2018) were performed on the MALT-derived taxonomic identifications.

CHAPTER 4: ASSESSING THE IMPACT OF INDUSTRIALIZATION ON THE FISHER-HUNTER-GATHER ORAL MICROBIOME USING A PAIRED ANCESTOR-DESCENDANT FRAMEWORK

Abstract

This chapter examines changes in the diet, health, and oral microbiome of the descendant Coast Tsimshian community. European colonization and subsequent settler-colonialism and industrialization represent another significant period of social transformation in Coast Tsimshian history. In other Indigenous North American communities, colonization and industrialization have been associated with dietary shifts away from traditional subsistence practices, toward a market food economy, which has increased reliance on starch and carbohydrate-rich foods. As these types of diets have been correlated with oral microbial dysbiosis and chronic inflammatory health conditions, investigating the relationship between dietary shifts associated with increasing industrialization and changes in oral microbial community composition may provide one explanation for how European colonization and subsequent industrialization has contributed to biological changes which result in negative health outcomes for Indigenous North American communities.

Introduction

European colonization and subsequent industrialization has led to dramatic changes in Indigenous subsistence due to removal from traditional lands and restricted access to traditional food sources (Willows 2005). As a result, in many First Nations communities market foods have overtaken traditional foods as primary contributors to the overall diet, driving a shift from traditional diets high in animal protein and low in fats and carbohydrates to alternative diets

higher in fats and sugars (Willows 2005). This shift from a stored food economy to a market food economy has been noted amongst the Coast Tsimshian (Martindale 2009). The Metlakatla First Nation continues to occupy parts of their traditional homeland, and many descendant community members still consume marine foods. However, factors such as cost and ease of access to traditional versus market foods, cultural changes, and modification of the physical environment may have reduced the community's reliance on marine foods (Willows 2005).

Indigenous communities face increased health challenges, such as higher rates of obesity, heart disease, and diabetes (IHS 2016; Willows 2005), in comparison to their non-Indigenous neighbors (Reading and Wien 2009). These inflammatory diseases have been correlated with a pathogenic shift in the oral microbiome, referred to as dysbiosis (Hartstra et al. 2015; Liu et al. 2012; Okuda et al. 2001; Slocum et al. 2016). The microbial-mediated relationship between high carbohydrate and sugar intake, associated with a Western/industrialized diet (Adler et al. 2013; Skelly et al. 2018), and increased rates of carious lesions and periodontitis is well documented (Duran-Pinedo and Frias-Lopez 2015; Soble 1994; Takahashi 2015). Therefore, the relationship between dietary change, oral health, and the oral microbiome may be one mechanism through which the cultural changes initiated by European colonization have contributed to biological changes which result in negative health outcomes for descendant populations (Skelly et al. 2018).

This chapter builds on the previous analyses of diet, health, and the oral microbiome of the ancestral Coast Tsimshian, comparing these results with analogous data from the descendant community of the Metlakatla First Nation. The collaborative research framework used in this study provides unique insight into the evolutionary relationship between diet and the oral microbiome. The ongoing research collaboration with Metlakatla has facilitated an analysis of

the shared patterns in microbial composition between the ancestral community and their genetic descendants, who continue to live in the same geographic environment. This represents a more rigorous approach than previous ancient and contemporary oral microbiome studies which have compared populations with different recent evolutionary histories, and points to unique adaptations in the Coast Tsimshian oral microbiome which have been retained despite dietary changes resulting from industrialization.

Results

Seventeen members of the Metlakatla First Nation volunteered to participate in the study by providing an oral gumline swab for genomic analysis, hair sample for isotope analysis, and completing a three-day food journal with an accompanying survey on demographic and health information. Eight of these participants currently live in Metlakatla (47.06%). Two-thirds (n=12) of the participants self-identified as female; four individuals identified as male. All individuals are over the age of eighteen.

Participants self-reported health history and lifestyle data which could potentially influence oral health and microbial composition. Half of the participants (total respondents n=12) reported taking oral antibiotics anytime during the year prior to sample collection. Eight participants (47.1%) indicated they had ever been diagnosed with a chronic disease, such as cancer, thyroid disease, rheumatoid arthritis, or seasonal allergies. Five of the seventeen participants (29.4%) reported they have been told by a doctor or health care provider that they have/had diabetes. Four participants (23.5%) indicated they currently smoke cigarettes.

Oral Health

Participants self-reported data on dental hygiene practices and several measures of dental health. Five descendant participants (35.7%) indicated they use dental floss (rarely, weekly, or daily). Seven individuals (41.2%) reported using mouthwash (rarely, weekly, or daily).

The incidence of carious lesions is higher amongst descendants than Ancestors. Only one descendant indicated they do not have any carious lesions (12.5% of respondents), in contrast to 93.3% of Ancestors who are caries-free. Amongst descendants, the mean percent of teeth with carious lesions is 27.8%. This is a significantly higher proportion of carious teeth than was observed in the Ancestors, who had a mean of 0.3% carious teeth, based on a t-test ($p=0.012$). In the descendant population, there was no significant relationship between the proportion of carious lesions and use of dental floss ($p=0.853$) or mouthwash ($p=0.934$). Whether or not an individual currently lived in Metlakatla was not significantly related to the prevalence of caries ($p=0.414$). Similarly, there was no significant relationship between the proportion of carious lesions and individual history of diabetes ($p=0.752$) or other chronic diseases ($p=0.390$), or use of oral antibiotics within the past year ($p=0.429$). However, individuals who reported they currently use tobacco did have a significantly lower proportion of dental cavities ($p=0.028$). Individuals who did not report current tobacco use had an average of 45.6% of teeth with carious lesions, while descendants who used tobacco at the time of data collection had a mean of 10.0% teeth with caries.

Only one participant reported they had been diagnosed with gingivitis in the year prior to data collection. Using a Fisher's Exact test, this is a significantly lower incidence of periodontal inflammation ($p<0.0001$) than in the ancestral population, where 93.3% of individuals exhibited periodontitis.

Five of sixteen descendant participants indicated they are edentulous (45.5%). Excluding these individuals, the descendant population has a significantly higher ($p=0.046$) rate of antemortem tooth loss, with an average of 26.6% of teeth lost, compared to the Ancestors, who lost an average of 11.5% of teeth. Within the descendant community, there was no significant relationship between the proportion of teeth lost antemortem and dental hygiene, health history, or behavioral data collected.

Isotopic analyses

Participants from the descendant community provided naturally shed whole-strand hair samples for analysis of $\delta^{15}N$ and $\delta^{13}C$ values from keratin (n=16). The $\delta^{13}C$ value of hair keratin is enriched by ~3‰ relative to diet (O'Connell and Hedges 1999), so the $\delta^{13}C$ values from the descendant hair samples were adjusted +2.1‰ to match the enrichment between diet and tooth collagen. This allows the isotope values from the hair keratin of descendants to be directly compared to the tooth collagen of Ancestors for dietary reconstruction.

Analysis of hair keratin provided $\delta^{15}N$ values representing the trophic level of the dietary protein consumed by each descendant. The $\delta^{15}N$ values obtained from keratin ranged from 7.7‰ to 10.8‰ across the descendant population. The mean $\delta^{15}N$ value of the descendants' keratin (9.6‰) was significantly lower than the value obtained from the Ancestors' collagen (19.3‰), indicated by a t-test ($p<0.0001$). A linear regression with a t-test indicates the proportion of teeth with carious lesions does not have a significant relationship to the $\delta^{15}N$ values measured in the descendant community ($p=0.452$), nor does the estimated proportion of antemortem tooth loss ($p=0.747$). A chi-squared logistic regression indicates there is also not a significant relationship between edentulism and $\delta^{15}N$ values ($p=0.057$). Whether or not a descendant lived in Metlakatla

at the time of data collection, rather than in Prince Rupert, was not significantly related to the $\delta^{15}N$ values measured (p=0.332).

Analysis of hair keratin also provided $\delta^{13}C$ values representing the relative marine or terrestrial components of the dietary protein consumed by each descendant. The $\delta^{13}C$ values obtained from keratin ranged from -18.3‰ to -16.4‰ across the descendant population. The mean $\delta^{13}C$ value of the descendants' keratin (-17.4‰) was significantly more negative than the value obtained from the Ancestors' collagen (-12.9‰), indicated by a t-test ($p<0.0001$). Neither the proportion of teeth with carious lesions (p=0.224), estimated proportion of antemortem tooth loss (p=0.086), or total tooth loss (p=0.456) was demonstrated to have a significant relationship to the $\delta^{13}C$ value of the descendants' keratin. Similarly, living in Metlakatla had no significant relationship to descendant $\delta^{13}C$ values (p=0.312). From the $\delta^{13}C$ keratin values, the estimated percent of marine source contribution to the dietary protein of the descendant community's diet ranged from 31.3% to 50.5%. The mean proportion of marine components in the descendants' dietary protein (40.5%) was significantly lower than that of the ancestral Coast Tsimshian community (84.9%) when evaluated via t-test ($p<0.0001$).

Microbiome

Oral microbiome metagenomes were successfully extracted from the gumline swabs of each of the 17 descendant community participants. Consistent with the analysis of samples from the Ancestors, the descendants' sequences were filtered to eliminate laboratory and reagent contaminants in the extraction and library blank controls. PERMANOVA test were used to identify possible confounding factors driving taxonomic diversity. As reported in Chapter 3, extraction group did not have a significant impact on microbial composition after filtering.

However, edentulous individuals did have a significantly different oral microbial composition than descendant participants with teeth ($p=0.042$). As all of the Ancestors sampled for this study had one or more teeth, the edentulous descendant participants were excluded from microbial analyses (remaining sample $n=11$). Statistical analyses mirroring those performed on the Ancestors' oral microbiome data are reported here for comparison.

Alpha diversity

Shannon's index was used to assess the abundance and evenness of microbial taxa within each sample. The results were statistically compared across samples using the Kruskal-Wallis test for pairwise analysis of categorical variables and Pearson correlation for continuous numeric variables. None of the variables predicted inter-individual differences in oral microbiota species richness and distribution (see Table 4.1).

Beta diversity

Bray-Curtis distance metrics, tested with PERMANOVA (categorical and ordinal variables) and Adonis (continuous variables), were used to identify statistically significant dissimilarity in microbial composition between samples. There was no statistically significant difference in oral microbiome composition between individuals with different dietary isotope compositions, oral hygiene or health, health history, or social factors (see Table 4.2).

Table 4.1. Tests of alpha diversity statistical significance				
Variable	**Statistical test**	***N***	**Groups**	***P* (<0.05)**
Social factors				
Sex (male/female)	Kruskal-Wallis	11	2	1
Currently living in Metlakatla	Kruskal-Wallis	11	2	0.221
Oral hygiene and health indicators				
Carious lesions (Presence/Absence)	Kruskal-Wallis	8	2	0.127
Carious lesions (Percent teeth affected)	Pearson	8	NA	0.398
AMTL (Presence/Absence)	Kruskal-Wallis	9	2	1
AMTL (Percent observable)	Pearson	9	NA	0.725
Gingivitis (Presence/Absence)	Kruskal-Wallis	11	2	0.114
Floss use	Kruskal-Wallis	11	2	0.144
Mouthwash use	Kruskal-Wallis	11	2	0.414
Recent antibiotic use	Kruskal-Wallis	8	2	0.083
Chronic disease history	Kruskal-Wallis	11	2	0.705
Diabetes history	Kruskal-Wallis	11	2	0.414
Current tobacco use	Kruskal-Wallis	11	2	0.257
Dietary isotope values				
$\delta^{13}C$ collagen	Pearson	11	N/A	0.129
$\delta^{15}N$ collagen	Pearson	11	N/A	0.760
Diet percent marine from collagen	Pearson	11	N/A	0.130

Table 4.2. Tests of beta diversity statistical significance				
Variable	**Statistical test**	***N***	**Groups**	***P* (<0.05)**
Social factors				
Sex (male/female)	PERMANOVA	11	2	0.580
Currently living in Metlakatla	PERMANOVA	11	2	0.799
Oral hygiene and health indicators				
Carious lesions (Presence/Absence)	PERMANOVA	8	2	0.632
Carious lesions (Percent teeth affected)	Adonis	8	NA	0.048*
Gingivitis (Presence/Absence)	PERMANOVA	11	2	0.243
Floss use	PERMANOVA	11	2	0.547
Mouthwash use	PERMANOVA	11	2	0.037*
Recent antibiotic use	PERMANOVA	8	2	0.659
Chronic disease history	PERMANOVA	11	2	0.789
Diabetes history	PERMANOVA	11	2	0.868
Current tobacco use	PERMANOVA	11	2	0.650
Dietary isotope values				
$\delta^{13}C$ collagen	Adonis	11	N/A	0.351
$\delta^{15}N$ collagen	Adonis	11	N/A	0.229
Diet percent marine from collagen	Adonis	11	N/A	0.338

Taxa overview

As a whole, the descendant microbiome is dominated by Actinobacteria (48.1%) and Firmicutes (26.8%), consistent with the oral microbiome of the ancestral Coast Tsimshian community (see Figure 4.1). These phyla have slightly elevated proportions compared to those observed in the ancestral microbiome. The phyla Proteobacteria (10.2%) is slightly reduced in abundance, while Bacteroidetes (6.8%) and Fusobacteria (5.8%) have slightly increased abundance, compared to the ancestral microbiome. The total number of phyla represented in the descendant microbiome is reduced compared to that of the Ancestors, as Euryarchaeota, Chloroflexi, and Synergistetes are not represented in the descendant microbiome. A PERMANOVA test of beta-diversity indicates there is a significant difference ($p=0.001$) in oral microbial diversity between the ancestral and descendant communities.

Significant dissimilarity was observed between descendants who did and did not use mouthwash, and between individuals with differing proportions of carious lesions. Individuals who used mouthwash at least once a week had elevated proportions of Actinbacteria and Proteobacteria, with reduced abundance of Firmicutes and Fusobacteria, and complete loss of Bacteroidetes (see Figure 4.2). There were no significant variation in the mean of individual taxa in relation to mouthwash use (FDR_P=1). While the proportion of carious lesions did drive significant variation in the descendant oral microbiome, there was no clear directional change in the relative abundance of taxa in relation to increasing proportions of carious lesions, which may be related to the small sample size (n=8). No individual taxa exhibited significant variation in relative abundance in relation to the presence or absence of carious lesions within the descendant population.

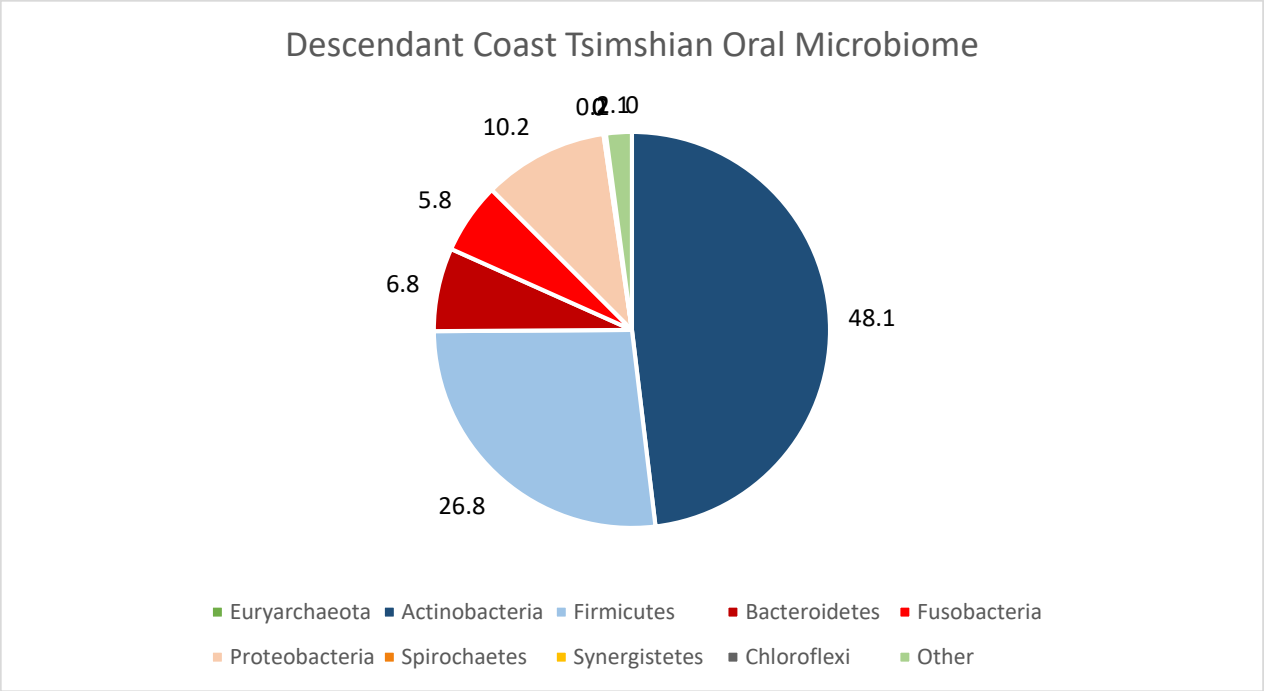

Figure 4.1. Relative abundance of bacterial phyla in the descendant Coast Tsimshian oral microbiome.

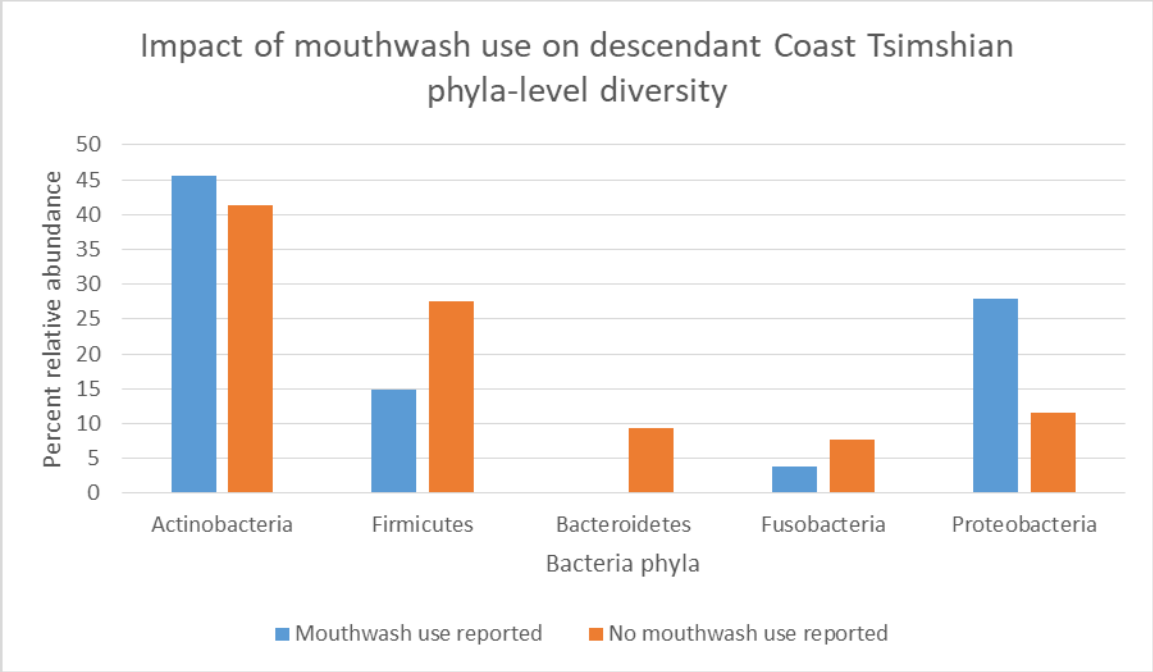

Figure 4.2. Comparison of relative abundance of bacterial phyla in the descendant Coast Tsimshian oral microbiome in relation to mouthwash use.

Correlation with oral health

To assess if the pattern of variation in microbial taxa related to oral pathologies observed in the ancestral Coast Tsimshian community continues in the descendant oral microbiome, an ANOVA test was employed to identify significant differences in abundance of individual taxa in relation to the presence or absence of gingivitis and carious lesions. However, there were no taxa within the oral microbiome of the descendant community that varied in abundance significantly in relation to the presence of these two pathologies (FDR_P = 1).

Discussion

The descendant Coast Tsimshian community continues to live in traditional Coast Tsimshian territory, occupying the same coastal environment as their ancestors. Isotopic analyses illustrate the degree to which descendant community members continue to rely on coastal food resources. Descendants obtain 31-51% of their dietary protein from marine sources, based on the $\delta^{13}C$ values obtained from their hair keratin. This is a lower proportion of marine dietary contributions than in the ancestral community, but demonstrates that marine protein remains a critical component of diet today. Overall, the ancestral community are consuming a higher trophic level of protein sources, more marine protein sources, than the descendant community. This is illustrated by the relatively high $\delta^{15}N$ and less negative $\delta^{13}C$ values derived from the Ancestors, which reflect a high reliance on marine mammals and higher trophic fish, like salmon, for dietary protein (see Figure 4.3). The $\delta^{15}N$ values of the descendant community are lower, indicating they are consuming some additional lower trophic proteins which are likely terrestrial, based on the more negative $\delta^{13}C$ values. These values reflect the integration of some new dietary resources in conjunction with European colonization and industrialization, but also

the continuing importance of marine subsistence in spite of the significant cultural and economic changes that have resulted from ongoing colonization and industrialization.

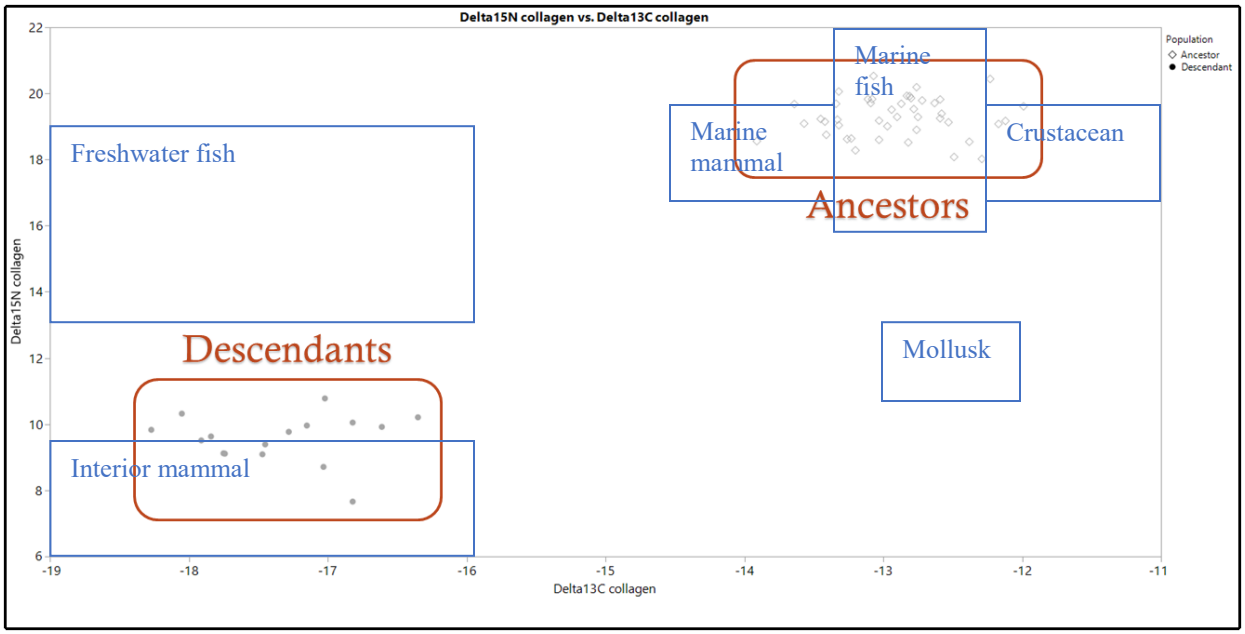

Figure 4.3. Comparison of collagen isotope values from Ancestors and keratin isotope values from the descendant community. Collagen isotope values of potential dietary resources in coastal British Columbia are included for reference from Schwarcz et al. (2014).

European contact, subsequent colonization, and industrialization have all been correlated with increased dental pathologies (Klaus and Tam 2010; Skelly et al. 2018; Adler et al. 2013). This pattern is partially reflected in the comparative data on oral health collected from Coast Tsimshian Ancestors and descendants. Descendants have increased proportions of teeth with carious lesions and increased antemortem tooth loss. However, descendants reported an extremely low rate of gingivitis. Based on these oral pathologies, it is expected the descendant oral microbiome would exhibit higher proportions of gram-negative bacteria, especially the red-complex bacteria associated with oral inflammatory conditions (Sudhakara et al. 2018).

Additionally, the caries-associated *S. mutans*, which was absent in the ancestral microbiome, may be present in the descendant oral microbiome, as it has been hypothesized to be associated specifically with industrialization in Europe (Adler et al. 2013), and may have been integrated in to the descendant Coast Tsimshian oral microbiome in conjunction with post-European contact genomic admixture and industrialization.

When comparing the oral microbiome generated from oral gumline swabs of descendant community members to those extracted from the dental calculus of the ancestral Coast Tsimshian community, there is a significant difference in the representation and abundance of bacterial phyla (see Figure 4.4). Similar to what has been reported in other studies of microbial community composition, there is a reduction in community diversity between the pre-industrial ancestral community, and the post-industrial descendant community (Adler et al. 2013; Zaura et al. 2017). However, the transition to a gram-negative dominated oral microbiome in the descendant population dominated by phyla like Bacteroidetes is not supported by the data from the Coast Tsimshian descendants. This community has retained the Actinobacteria and Firmicutes dominant taxonomic composition exhibited by their ancestors.

The oral microbiome taxonomic composition may not be directly comparable between the ancestral and descendant communities, because of the difference in sample types. Zaura et al (2009) has demonstrated that variation in microbial community composition exists between oral sampling sites. While the bacterial DNA extracted from the dental calculus of the Ancestors reflects an anaerobic plaque environment, the oral gumline swabs from descendant community members may more closely reflect the salivary oral microbiome than the microbiome of a plaque community.

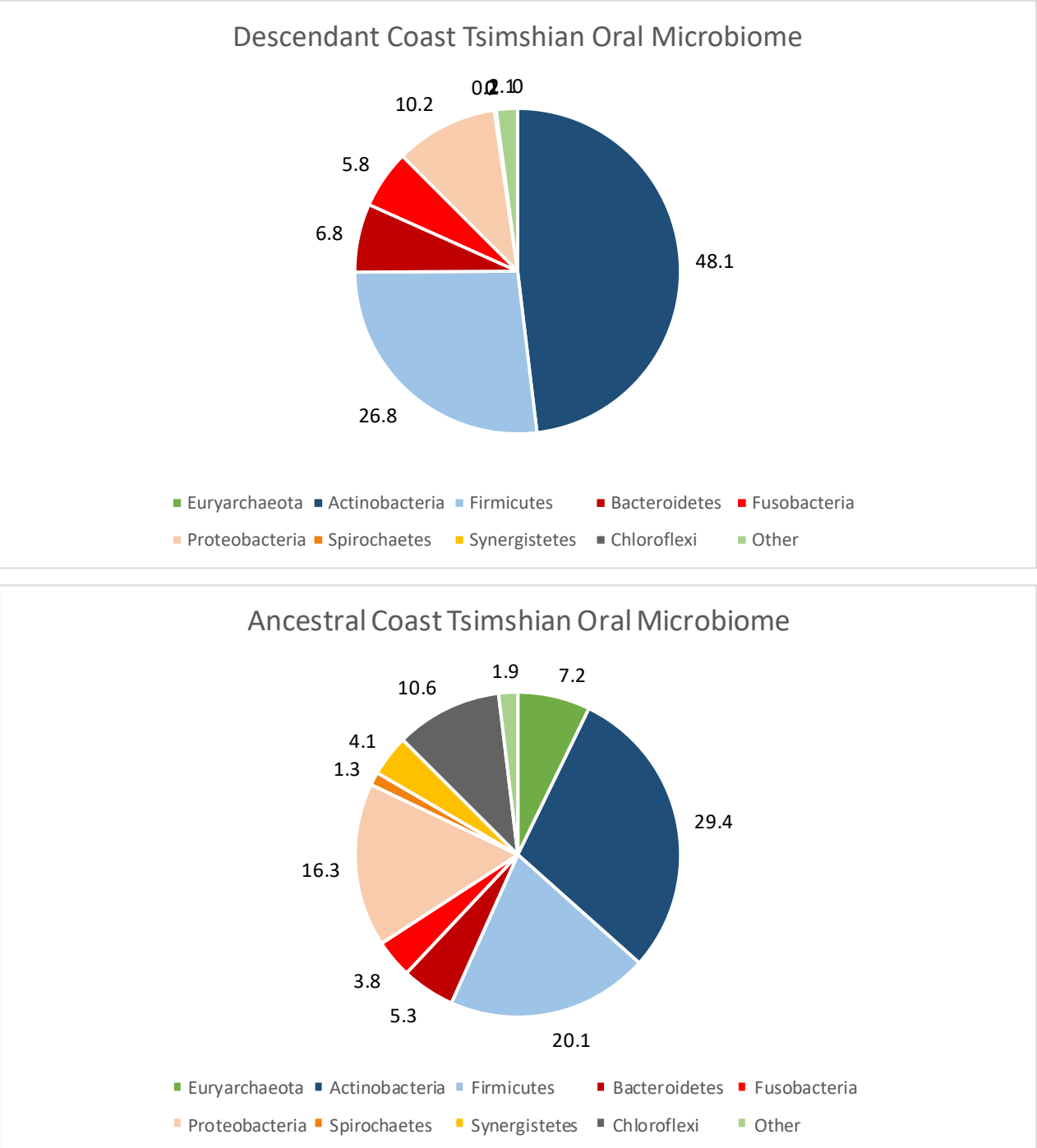

Figure 4.4. Comparison of phyla-level taxonomic composition of the ancestral and descendant Coast Tsimshian oral microbiomes.

However, when comparing the oral microbiome of the Coast Tsimshian descendant community to published results from other post-industrial Indigenous North American community, there is a clear difference in the dominant phyla represented. While the descendant Coast Tsimshian community have an oral microbiome dominated by Actinobacteria and Firmicutes, Ozga et al. (2016) found the oral microbiome of Cheyenne and Arapaho communities in Oklahoma were dominated by Bacteroidetes, with very low abundance of Actinobacteria. The non-Indigenous comparative sample group from Oklahoma was dominated by Firmicutes, with relatively equal abundances of Actinobacteria, Proteobacteria, and Bacteroidetes (Ozga et al. 2016). Zaura et al.'s (2009) "healthy" core salivary microbiome is dominated by Firmicutes, with secondary contributions from Actinobacteria and Proteobacteria, and reduced abundance of Bacteroidetes.

The consistent dominance of Actinobacteria across the ancestral and descendant microbiomes of the Coast Tsimshian communities, despite differences in sample types, suggests the microbiome the Ancestors developed in combination with a high-protein fisher-hunter-gatherer lifestyle, has been retained within the descendant community despite the dietary changes associated with industrialization indicated by the isotope values from the descendant hair keratin. Despite the reduced proportion of high-trophic marine protein in the descendant community diet, the microbiome of the descendant community continues to compositionally reflect a fisher-hunter-gatherer subsistence strategy. This may highlight the importance of Indigenous communities maintaining access to and consumption of traditional foods in order to prevent the shift to gram-negative phyla exhibited in other industrialized populations. The consistent low abundance of gram-negative phyla like Bacteroidetes, despite the high prevalence of oral pathologies observed in both the descendant and ancestral communities, points to another

unique aspect of the Coast Tsimshian oral microbiome. The Actinobacteria dominated oral microbiome exhibited by the ancestral Coast Tsimshian and retained by the descendant community may be a particularly resilient oral microbiome compositional structure. Functional analyses of these oral bacterial communities is necessary for further understanding of how the unique microbial taxonomic composition of the coast Tsimshian oral microbiome may positively support the descendant community's biological resilience in the wake of European colonization and industrialization.

The paired Ancestor-descendant research framework employed here to assess changes in diet, health, and the oral microbiome in Coast Tsimshian communities has provided unique insight into the potential adaptation of the human oral microbiome to a fisher-hunter-gatherer subsistence strategy, and its resilience despite cultural and economic changes associated with European colonization and industrialization. Isotopic data has illustrated that the marine-focused diet of the ancestral Coast Tsimshian community has been maintained, to varying degrees, by the descendant community, and this may have facilitated the retention of a gram-positive dominated oral microbiome, even as the prevalence of carious lesions and antemortem tooth loss has increased. Future analyses of the functional composition of the ancestral and descendant microbial data may shed additional light on the marine-adapted traits of the Coast Tsimshian oral microbiome.

Materials and methods

Food journals/survey

Each descendant was asked to complete a three day food journal and a health and demography survey. Data on oral health was extracted from these surveys. The proportion of

teeth with carious lesions was estimated as the total number of filled and unfilled cavities and root canals reported, divided by the number of natural teeth reported. The proportion of teeth lost antemortem was estimated from the number of extracted permanent teeth reported. Participants self-reported if they had been told by a medical professional within the year prior to data collection that they had gingivitis.

Isotope purification and analysis

Keratin was extracted from 1-2mg whole-strand hair samples freely shed via brushing and collected by participants. A total of 16 participants provided hair samples. To purify, the samples were sonicated in petroleum ether for five minutes three times, with the ether decanted and replaced between each sonication. The ether was decanted and the samples rinsed clean with distilled water. The cleaned samples were placed in a freeze-drier overnight. The cleaned and dried hair strands were then chopped into approximately 1mm size pieces, suspended in distilled water, and frozen in a conventional freezer before being transferred to the freeze drier. The dried hair samples were transported to the Illinois State Geological Survey Stable Isotope Lab for isotopic analysis using a Carlo-Erba NC 2500 elemental analyzer connected to a Thermo Finnigan Conflo IV universal continuous flow interface and Thermo Finnigan Delta V Advantage isotope ratio mass spectrometer. Analysis of the hair keratin provided measures of $\delta^{13}C$ and $\delta^{15}N$.

DNA collection, extraction, and sequencing

OMNIgene ORAL swab kits (DNA Genotek OMR-110) were used to collected oral microbiome samples. The samples were extracted using the Epicentre MasterPure Complete

DNA and RNA Purification Kit following protocol PD-PR-00554. Two extraction blank controls/negatives were extracted from unused swab kits.

A genomic library was built from each extracted DNA sample and the two extraction blank controls using the NuGEN Ovation Ultralow Library Prep kit with Illumina-compatible unique dual indexes. Each extract was diluted to 100ng or less of DNA per library build. An additional water library was also constructed as a final negative control. The libraries were amplified with Phusion HS II for 7-15 cycles, depending on the quantity of DNA in the starting extract. All negative libraries were amplified for 15 cycles.

The amplified libraries were purified using an Agentcourt AMPure bead clean-up. The purified libraries were confirmed via gel electrophoresis and the DNA concentration quantified before being quality checked on an AATI Fragment Analyzer at the Roy J. Carver Biotechnology Center at UIUC. A final size selection was performed prior to sequencing. Each library was sequenced on the Illumina HISEQ 4000, producing 150bp single-end reads.

Bioinformatic analyses

The sequencing produced 168-20 million DNA sequences per dental calculus sample. The sequences were quality trimmed, the adapter removed, and filtered to a minimum length of 50bp before taxonomic assignment. Each set of trimmed sequences was subsampled to two million sequences and aligned to the RefSeqGCS database using MALTn (Herbig et al. 2016) nucleotide alignment (Eisenhofer and Weyrich 2019). The resulting taxonomic classifications were imported into MEGAN6 (Huson et al. 2016) and visually assessed before all extraction and library controls were subtractively filtered from the taxonomic classification results.

Downstream statistical analyses and visualization of microbial community composition was performed in QIIME (Caporaso et al. 2010) and QIIME2 (Bolyen et al. 2018).

CHAPTER 5: CONCLUSION

This study has presented a much-needed characterization of the oral microbiome of an ancestral fisher-hunter-gatherer community. The collaborative research relationship with the Metlakatla First Nation has facilitated a unique paired ancestor-descendant comparison that has illuminated the resilience of this fisher-hunter-gatherer microbiome over time, despite significant changes in diet and oral health associated with periods of social transformation throughout Coast Tsimshian history. This study also highlights the need for additional research, following the model presented here, to address the ascertainment bias in oral microbiome research which may be shaping how bacterial taxa are interpreted to be related to disease-causing processes, resulting in populations underrepresented in the microbial research literature being erroneously pathologized.

The integration of osteological, genomic, and isotopic data in has supported the hypothesized relationship between social stratification, inter-individual dietary variation, and osteological indicators of health inequality in the ancestral Coast Tsimshian community. Novel isotopic data derived from the dental apatite of Ancestors revealed dietary variation driven by status, which was interpreted with the aid of community-held knowledge shared by representatives from the descendant community. Additionally, changes in diet related to intensification of food processing and storage corresponded to the emergence of non-egalitarian social complexity between the Middle Pacific and Late Pacific periods. While there were variations in oral health related to diet, there was no direct relationship between skeletal and dental evidence of health inequality and the indicators of social stratification. The data presented in this chapter has contributed a new, individual-level, biological perspective to understanding

the impact of the increasing social complexity on individuals within the ancestral Coast Tsimshian community.

Building on these analyses, the ancient DNA sequenced from the dental calculus of Coast Tsimshian Ancestors has provided a much-needed characterization of the oral microbiome of an ancestral fisher-hunter-gatherer community. Inter-individual differences in diet related to status and social change between the Middle and Late Pacific periods was not mirrored in the oral microbiome taxonomic composition, indicating the oral microbiome may not be an accurate reflection of inter-individual social inequality in resource-rich populations with minimal inter-individual dietary variation. As a whole, the oral microbiome of the ancestral Coast Tsimshian community reflects their high-protein, marine-focused fisher-hunter-gatherer subsistence strategy.

Within the ancestral community, variation in mean abundance was observed in taxa correlated with skeletal evidence of periodontitis and carious lesions. While the bacterial species associated with the presence of carious lesions were analogous to the gram-positive, acid-producing bacteria most commonly associated with carious lesions in the clinical literature, the bacteria associated with periodontitis absence was not consistent with previous clinical descriptions. The lack of Bacteriodetes in the ancestral Coast Tsimshian oral microbiome, despite high prevalence of periodontitis, alveolar abscesses, and antemortem tooth loss, may point to a specific adaptation of the ancestral oral microbiome during their evolutionary history which has suppressed the abundance of Bacteroidetes even in association with inflammatory oral environments.

The paired Ancestor-descendant comparative research framework employed in this study has highlighted the resilience of the Coast Tsimshian oral microbiome despite change in diet and

oral health related to ongoing colonization and increasing industrialization. The oral microbiome of the descendant Coast Tsimshian community remains dominated by Actinobacteria and Firmicutes, with low abundance of Bacteroidetes despite a high prevalence of antemortem tooth loss, in clear contrast to other post-industrial Indigenous and non-Indigenous North American communities. The isotopic analysis of hair keratin provided by descendant community participants indicates that while the consumption of marine dietary protein is reduced in the descendant community, it remains a significant dietary resource. Maintaining elements of a fisher-hunter-gatherer diet may serve a protective function against the shift in oral microbial composition toward gram-negative phyla exhibited in other post-industrial communities, highlighting one potential strategy for biological resilience in Indigenous North American communities.

Future research will explore the functional composition of the ancestral and descendant Coast Tsimshian oral microbiomes. This research may provide insight in to how the high abundance Actinobacteria and Firmicutes microbiome exhibited by the Coast Tsimshian communities is functionally adaptive in relation to marine-based subsistence. Additionally, these analyses may improve understanding of how the oral microbial community is functionally responding to inter-individual variation in diet and health. This research will further address the ascertainment bias in human oral microbiome research which has failed to account for the impact of population-specific recent evolutionary histories and adaptations when characterizing oral microbial diversity, especially in the contexts of health and disease.

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APPENDIX A: GENOMIC READS USED IN DIETARY ANALYSES

Sample Identifier	Sample type	Sequencing platform	# Sequence reads generated (trimmed, quality and minimum length filtered)	# Deduplicated reads used for analyses
BC-28	Ancestor (calculus)	HiSeq 4000	20230894	8156768
BC-34	Ancestor (calculus)	HiSeq 4000	168087953	106898721
BC-46	Ancestor (calculus)	HiSeq 4000	43126662	12412498
BC-140	Ancestor (calculus)	HiSeq 4000	26368403	9123823
BC-312	Ancestor (calculus)	NovaSeq 6000	29938544	19720035
BC-319	Ancestor (calculus)	NovaSeq 6000	38415770	23100553
BC-321	Ancestor (calculus)	NovaSeq 6000	33571511	21489597
BC-339	Ancestor (calculus)	NovaSeq 6000	37234440	23819990
BC-347	Ancestor (calculus)	NovaSeq 6000	37709068	23466931
BC-347rep	Ancestor (calculus)	HiSeq 4000	22040340	17781457
BC-364	Ancestor (calculus)	NovaSeq 6000	33536680	20709472
BC-378	Ancestor (calculus)	NovaSeq 6000	39840433	24540146
BC-380	Ancestor (calculus)	NovaSeq 6000	34799228	21145111
BC-382	Ancestor (calculus)	NovaSeq 6000	32696561	17052756
BC387	Ancestor (calculus)	NovaSeq 6000	28585755	17772058
BC-388	Ancestor (calculus)	HiSeq 4000	44285419	23625559
BC-388rep	Ancestor (calculus)	HiSeq 4000	37190228	25006325
BC-406	Ancestor (calculus)	NovaSeq 6000	45336438	29466345
BC-409	Ancestor (calculus)	NovaSeq 6000	42572575	25093416

Sample Identifier	Sample type	Sequencing platform	# Sequence reads generated (trimmed and quality filtered)	# Deduplicated reads (used in dietary analyses)
BC-410	Ancestor (calculus)	NovaSeq 6000	41997422	21091603
BC-411	Ancestor (calculus)	NovaSeq 6000	51291024	28118524
BC-411rep	Ancestor (calculus)	HiSeq 4000	34039842	23925022
BC-412	Ancestor (calculus)	NovaSeq 6000	46056708	28666551
BC-446	Ancestor (calculus)	NovaSeq 6000	51952522	32777073
BC-446rep	Ancestor (calculus)	HiSeq 4000	28656855	23158879
BC-447	Ancestor (calculus)	HiSeq 4000	37799597	20892896
BC-450	Ancestor (calculus)	NovaSeq 6000	33569010	19551993
BC-453	Ancestor (calculus)	NovaSeq 6000	34092813	17178372
BC-455	Ancestor (calculus)	NovaSeq 6000	38388976	25485552
BC-458	Ancestor (calculus)	NovaSeq 6000	37635556	24223139
BC-459	Ancestor (calculus)	NovaSeq 6000	48324050	26624884
BC-461	Ancestor (calculus)	NovaSeq 6000	44637290	17755008
BC-463	Ancestor (calculus)	NovaSeq 6000	35999013	21496424
BC-473	Ancestor (calculus)	NovaSeq 6000	35126430	21932044
BC-474	Ancestor (calculus)	HiSeq 4000	44846054	27005576
BC-474rep	Ancestor (calculus)	HiSeq 4000	41223796	31025000
BC-475	Ancestor (calculus)	HiSeq 4000	39242099	22835544
BC-475rep	Ancestor (calculus)	HiSeq 4000	37053455	27806809
BC-476	Ancestor (calculus)	NovaSeq 6000	37041742	23197353
BC-489	Ancestor (calculus)	NovaSeq 6000	35505689	15630462

Sample Identifier	Sample type	Sequencing platform	# Sequence reads generated (trimmed and quality filtered)	# Deduplicated reads (used in dietary analyses)
BC-495	Ancestor (calculus)	HiSeq 4000	55058812	27271145
BC-495rep	Ancestor (calculus)	HiSeq 4000	51291638	32088723
BC-496	Ancestor (calculus)	NovaSeq 6000	32973384	21472216
BC-501	Ancestor (calculus)	HiSeq 4000	33844271	21930002
BC-502	Ancestor (calculus)	HiSeq 4000	34518293	21953300
BC-504	Ancestor (calculus)	NovaSeq 6000	38150725	23527528
BC-520	Ancestor (calculus)	NovaSeq 6000	39029630	22793797
BC-525	Ancestor (calculus)	NovaSeq 6000	33900945	11596652
BC-886	Ancestor (calculus)	NovaSeq 6000	40871675	21685594
BC-886rep	Ancestor (calculus)	HiSeq 4000	25192155	17374609
BC-889	Ancestor (calculus)	NovaSeq 6000	36874498	12663100
BC-892	Ancestor (calculus)	NovaSeq 6000	36032518	20574167
BC-893	Ancestor (calculus)	NovaSeq 6000	35145273	17261635
Neg-B	Negative control	NovaSeq 6000	44927916	6501237
Neg-C	Negative control	HiSeq 4000	6391526	1967161
Neg-D	Negative control	NovaSeq 6000	47490587	5380124
Neg-E	Negative control	NovaSeq 6000	54452947	7790227
Neg-F	Negative control	NovaSeq 6000	45766367	4768516
Neg-L	Negative control	HiSeq 4000	13158	2684
Neg-S	Negative control	HiSeq 4000	5261511	1206614
Neg-X	Negative control	NovaSeq 6000	50880161	7453503

Sample Identifier	Sample type	Sequencing platform	# Sequence reads generated (trimmed and quality filtered)	# Deduplicated reads (used in dietary analyses)
BC-4610	Soil	HiSeq 4000	25997088	10636929
BC-5268	Soil	HiSeq 4000	27533658	14802766
Neg-1m	Negative control	HiSeq 4000	28758	N/A
Neg-2m	Negative control	HiSeq 4000	23018	N/A
Neg-H20m	Negative control	HiSeq 4000	5864	N/A
BC-01m	Descendant (oral swab)	HiSeq 4000	26980990	N/A
BC-02m	Descendant (oral swab)	HiSeq 4000	28488499	N/A
BC-03m	Descendant (oral swab)	HiSeq 4000	34841610	N/A
BC-04m	Descendant (oral swab)	HiSeq 4000	38027242	N/A
BC-05m	Descendant (oral swab)	HiSeq 4000	31399125	N/A
BC-06m	Descendant (oral swab)	HiSeq 4000	28068037	N/A
BC-07m	Descendant (oral swab)	HiSeq 4000	31564452	N/A
BC-08m	Descendant (oral swab)	HiSeq 4000	32583685	N/A
BC-09m	Descendant (oral swab)	HiSeq 4000	24642527	N/A
BC-10m	Descendant (oral swab)	HiSeq 4000	30002199	N/A
BC-11m	Descendant (oral swab)	HiSeq 4000	28000450	N/A
BC-12m	Descendant (oral swab)	HiSeq 4000	34616217	N/A
BC-13m	Descendant (oral swab)	HiSeq 4000	32550496	N/A
BC-14m	Descendant (oral swab)	HiSeq 4000	31951614	N/A
BC-15m	Descendant (oral swab)	HiSeq 4000	33527132	N/A
BC-16m	Descendant (oral swab)	HiSeq 4000	31791499	N/A
BC-17m	Descendant (oral swab)	HiSeq 4000	32651690	N/A

APPENDIX B: FILTERING OF GENOMIC READS USED IN MICROBIOME ANALYSES

Filtering applied	**Count of taxa assigned**	**Number of samples included**
None	80764377	83
All negative controls removed	50229560	72
Only soil samples removed	61195716	81
All control samples removed (negative controls and soil samples)	49881258	70
All controls and replicate samples removed	43175041	62
Filtered data used in QIIME/2 analyses	**Count of taxa assigned**	**Number of samples included**
All Ancestors	45619451	53
Ancestors with replicates excluded	38913234	45
All descendants	4261807	17